The Effect of Storage at -70°C and -150°C on the Torsion Properties of the Canine Femur

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Abstract

This study investigates the effect of storage at-150 °C in the vapourphase of liquid nitrogen on the torsion properties of whole intact femurs to be used as allografts. Twenty-five adult dog femurs, stripped of all soft tissue, were used. It has been established that storage at - 70 °C does not significantly affect the torsion properties of bone. In this study we found no significant difference in structural properties in torsion between the allografts stored at -150 °C and - 70 °C. However, cortical hairline cracks, which were not present before storage, after the bones were stored for 3 months and thawed at 1 °C/min rate were observed. These cracks fractured longitudinally, and not spirally, when tested to failure and were noted to originate from the vessel foramen. The mean torsion strength of these "damaged" bones was reduced by 48%. We also determined that these hairline cracks occurred during the thawing process at about -42 °C. This may suggest an effect of recrystallisation of fluids or water crystals within the "closed" medullary cavity and the foramen, thus increasing the pressure and paying for hairline cracks to propagate from stress risers. No incidence of cortical hairline cracks were observed if the medullary canal was decompressed and the medullary canals washed-out.

In tissue banking whole intact bones as allografts, caution should be taken when selecting the freezing and thawing rates for storage as cortical hairline cracks could result and eventually weaken the strength of the allograft.

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Key words: Allograft transplantation, Cryopreservation, Effects of freezing and cooling rates, Liquid nitrogen, Microscopic cracks, Stress risers, Torsion properties

Introduction

Allograft transplantation of bone has been established as a viable and useful reconstructive procedure when limb salvage is the option of choice.[1-5] For bone allografts, this is thought to be due to the relatively avascular structure and that low-freezing or freeze-drying for periods beyond three months reduces cellular immunogenicity.[6-9] However, in the storage of tissue for transplantation, the transmission of viral and bacterial diseases, like hepatitis-B virus (HBV), human immuno-deficiency virus (HIV) and slow virus infections, is a concern. Although outside the body such organisms are rapidly destroyed, it has known to be viable in highly cellular bone marrow present in cancellous bone stored at -80°C.[10-12] An alternative is the very-low temperature storage of tissue using the vapour phase of liquid nitrogen.

As the primary function of the transplanted bone is to restore anatomical and structural integrity to a weight-bearing limb, some workers[13-16] have shown that low freezing between -20°C and -80°C has little effect on the mechanical properties of long bones. Pelkar et al[14] also showed that ultra low freezing in liquid nitrogen at -196°C has no effect on the torsion strength and the energy to failure of rat femurs. However, they found that the torsion strain was higher and the torsion stiffness lowered. This would suggest a change in the stiffness of the collagen matrix with the strength of the hydroxyapatite bonding remaining unchanged. They also demonstrated that when the bones were freeze-dried there was a loss of torsion strength. This was due to the presence of micro-cracks in the bone that occurred during the rehydration process and was observed along the longitudinal axis of the specimen.

In this study, we investigated the effects of ultra-low temperature (-150°C) storage on the mechanical properties of long bone in a larger animal model. We also

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studied the occurrence of hairline cortical cracks during the freezing and thawing process.

Materials and Methods

Specimen *preparation*: Twenty-five adult mixed-bred dogs were used. The whole intact femur, sub-periosteally stripped of all soft tissue, was chosen as the model. Visual inspection was made for defects of the cortex and for the location of the nutrient artery foramen, which is usually situated at the junction of the proximal and distal third of the femur. For each dog, one femur was randomly selected and stored in -70°C in a standard electrical-run freezer while the contra-lateral femur was stored at -150°C in the vapour phase of liquid nitrogen, in a liquid nitrogen freezer (XLC 440, MVE Cryogenics, New Prague, Minnesota). All bones were first wrapped in a dry gauze and then in a cotton drape and placed in hermetically-sealed transplant bag (Ziploc™, Dow Chemicals, USA). This was to ensure that no water permeated through and that it remained dry within the bag. Storage of the specimens was over a period of three months. Platinum temperatures probes were used to continuously monitor the temperatures in both freezers using a two-pen analog recorder (SA-100P, Sekonic, Tokyo, Japan). The paired bones were divided into three groups (Table I). When the bones were placed into the freezer directly it was considered as uncontrolled freezing.[17] Uncontrolled freezing of the bone within the storage package as described above (14 to 25°C/min). Similarly, when it was taken out directly from the freezer to ambient room temperature, this was considered as uncontrolled thawing, measured between 8°C/min and 15°C/min. Controlled freezing-and thawing were done at rates of 1°C/min using a microprocessor temperature-controlled chamber (Kryo Series Planer Products, Sunberry-on-Thames, England). As there is a potential for changes in the internal pressures in the medullary canal which could have an effect on the integrity of the bone during the freezing and thawing processes, Group I was repeated in Group II with an additional step of decompressing the medullary canal by drilling a 10-mm diameter cavity at the intracondylar notch of the distal femur. This was 10-mm anterior to the femoral attachment of the anterior cruciate ligament. This location was chosen so that the integrity of the bone cortex would not be compromised, yet allowing the decompression of medullary canal by a wash-out of the canal. Group III looked at the effect of controlled freezing and thawing at 1°C/min. As we had observed the presence of cortical cracks occurring with the controlled thawing, we did an additional set of 5 samples (Group IVA). In this group the controlled thawing was done at 2°C/min for femurs stored at -150°C. This was to determine if increasing the thawing rate would have an effect. We also did another supplement group to test the effect of uncontrolled freezing and thawing at -70°C. This was more as a control, to observe for cortical hairline cracks during the storage process.

Torsion test: After thawing to room temperature and prior to mechanical testing, anterior-posterior and lateral radiographs of the paired femora were taken. Visual inspection was also made to check for the presence of cortical defects and cracks. Measurements of the mid-shaft size in the anterior-posterior direction were also taken as a measure of the external diameter (d_o) at the mid-shaft of the femur. The thickness (t) and the internal diameter at the mid shaft was also measured (d_i) from the radiographs. The gauge length for testing the torsion properties of the diaphyseal bone of the femur was set at 60-mm. This was to ensure a relatively uniform cross-section along the gauge length. The proximal end of the femur was first potted into self-curing dental cement (Vertex, Dentimax, Zeist, Holland) in a fixture. The mid-shaft was aligned to the axis of the fixture (Intrade, Singapore). The proximal end of the fixture was then clamped to the moving cross-head of the universal test-

TABLE I: FOR EACH PAIR OF FEMURS FROM THE SAME ANIMAL, ONE WAS RANDOMLY SELECTED AND STORED IN -70°C (MECHANICAL FREEZER) WHILE THE OTHER WAS STORED IN -150°C (VAPOUR PHASE OF LIQUID NITROGEN). ALL SPECIMENS WERE STORED FOR 3 MONTHS PRIOR TO THAWING.

Group	A (-70°C, mechanical freezer)	B (-150°C, vapour phase of liquid nitrogen)
I (n = 5)	Uncontrolled freeze Controlled thaw to RT at 1°C/min	Uncontrolled freeze Controlled thaw to RT at 1°C/min
II (n = 5)	Decompression of medullary canal Uncontrolled freeze Controlled thaw to RT at 1°C/min	Decompression of medullary canal Uncontrolled freeze Controlled thaw to RT at 1 °C/min
III (n = 5)	Controlled freeze at 1 °C/min Controlled thaw to RT at 1°C/min	Controlled freeze at 1 °C/min Controlled thaw to RT at 1 °C/min
Supplement Group IV (n = 5)	Uncontrolled freeze Uncontrolled thaw to RT	Uncontrolled freeze Controlled thaw to RT at 2°C/min

RT: room temperature (= 24°C)

ing machine (Autograph DCS-5000, Shimadzu, Kyoto, Japan) and the longitudinal axis of the shaft aligned to the axis of the lower fixture and potted in dental cement. The specimens were tested in external rotation at 0.18 rpm. A 50-kgf.m load cell was used and a force-time chart recorder (Dateletty 401, Shimadzu, Kyoto, Japan) recorded the load-displacement curves. All bones were tested to failure.

Specimen *analysis*: As the fractures were brittle with little deformation, specimens were put back together, secured and sectioned in the plane perpendicular to the axis of the medullary canal. The mean cross sectional area A_c, and the polar area moment of inertia, J was determined from the mean inner (d_i) and outer (d_o) diameter of the mid-shaft of the femur. This was also used to confirm the diameter measurements taken from radiographs.

Cross-sectional Area, $A, = \pi (d_o^2 - d_i^2) / 4 \ (m^2)$ and

Polar area moment of Inertia, $J = \pi (d_o^4 - d_i^4) / 32 \ (m^4)$

The structural behaviour was defined from the peak torque-twist values (ultimate torque) M, and stiffness slopes (torsion stiffness) S, were obtained from the charts. The material behaviour was defined from the maximum shear stress due to torsion or simply the maximum torsion stress, τ which was determined from the following equation.

Maximum torsion stress, $\tau = M r_o / J \ (MPa)$

where r_o, is the mean outer radius and J is the polar area moment of inertia.

The mean maximum torsion stress from the paired specimens was presented as a percentage of the control. The Wilcoxon signed-rank test was used to determine significance. Groups were treated independently after it was established that their geometric properties were similar. These were then compared against each other using a one-way analysis of variance to determine the effect of differences in the storage process for the mechanical freezer (Groups IA, IIA, IIIA, and IVA) and the liquid nitrogen freezer (Groups IB, IIB, IIIB and IVB) on the mechanical properties. All tests were at a 0.05 level of significance.

Results

Geometric properties: The paired bones were assessed for variation in their geometric properties at the mid-shaft and were found not to be significantly different (Table II). The mean polar moment area of inertia of all bones was $(3.87 \pm 1.3) \times 10^{-9} m^4$, the mean thickness at the mid-shaft of all bones was $(2.58 \pm 0.37) \times 10^{-3}$ m, while the mean outer (periosteal) diameter of at the mid-shaft of all bones was $(14.9 \pm 1.28) \times 10^{-3}$ m.

Cortical hairline cracks: Visual inspection and post-freezing radiographs prior to testing clearly identified in some of the bones, prominent longitudinal hairline cracks that were not present in the pre-storage radiographs (Table III). It was noted that the groups that were frozen (uncontrolled) and then with controlled thawing (i.e. Group IA, IB and IVB) had a higher occurrence of cortical hairline cracks resulting after the storage process. Group I had at least one of all its pairs of femora exhibiting cortical cracks prior to mechanical testing. However, with the decompression of the medullary canal (i.e. Group IIA and IIB), there were no occurrence of such cracks. Using a Fisher's exact test for 2 x 2 tables, decompression was found to significantly reduced the occurrence of cracks for storage at -70°C ($P = 0.023$), but was not significant for bones stored at -150°C ($P = 0.22$), probably due to its sample size. For storage at -70°C, the controlled freeze rate at 1°C/min compared to uncontrolled freeze rate, had a reduced chance for cracks to occur ($P = 0.023$) when both were thawed at 1°C/min. This was not significant for bones store at -150°C ($P = 0.09$), again because of its sample size. No other significant relationship between the two storage temperatures or the rate of freezing and thawing could be determined.

Those with cortical hairline cracks were noted to have the cracks originated from the foramen of the nutrient vessels extending proximally and distally for varying lengths along the axis of the medullary canal (Fig. 1).

TABLE II: DIFFERENCE IN GEOMETRY OF THE PAIRED BONES

	n (pairs)	Mean cortical thickness (mm)	Mean outer (periosteal) diameter (mm)	Mean inner (endosteal) diameter (mm)
Mean difference between the paired samples	25	0.07 ± 0.31	0.00 ± 0.27	0.05 f0.45
P value (paired t-test)		0.32	0.99	0.59
Statistical significance		ns	ns	ns

ns: not significant

TABLE III: OBSERVATION OF CORTICAL HAIRLINE CRACKS AFTER THE STORAGE PROCESS

Groups	Cortical cracks present	Cortical cracks absent
IA P = 0.023	4	
IB *P = 0.023*	2	3
IIA	*0*	5
IIB	0	5
IIIA	0	5
IIIB	1	4
IVA	1	4
IVB	2	3

A Fisher's Exact test for 2 x 2 tables was used to determine differences between groups on the occurrence of cracks.

Torsion test: The pairs in which neither had cortical cracks during the thawing process were tested for their torsion properties. The results are summarised in Table IV. The Wilcoxon signed-ranked non-parametric analysis found that there were no significant difference in their structural properties, viz. mean maximum torque, mean maximum angular rotation and mean elastic torsion stiffness. When the groups were tested independently no significant differences were noted between the different storage processes for their material behaviour (Table V). All bones with no cracks observed after the storage process sustained spiral fractures when tested to failure. These may or may not have originated from the nutrient artery foramen (Fig. 2A).

Specimen pairs, with at least one specimen that had a cortical hairline during the storage process, were considered separately but tested in the same manner (Table VI). The specimens with the cortical cracks failed with longitudinal fractures which propagated along the middle third of the diapyhysis initiating from the nutrient vessel foramen (Fig. 2B). There were no significant differences in the polar moment of inertia indicating comparable architecture properties between the paired bones in each group. However, there was approximately 48% reduction in maximum torsion stress as a result of a pre-existing longitudinal hairline crack, which in structural terms are stress risers rather than a longitudinal section defect.

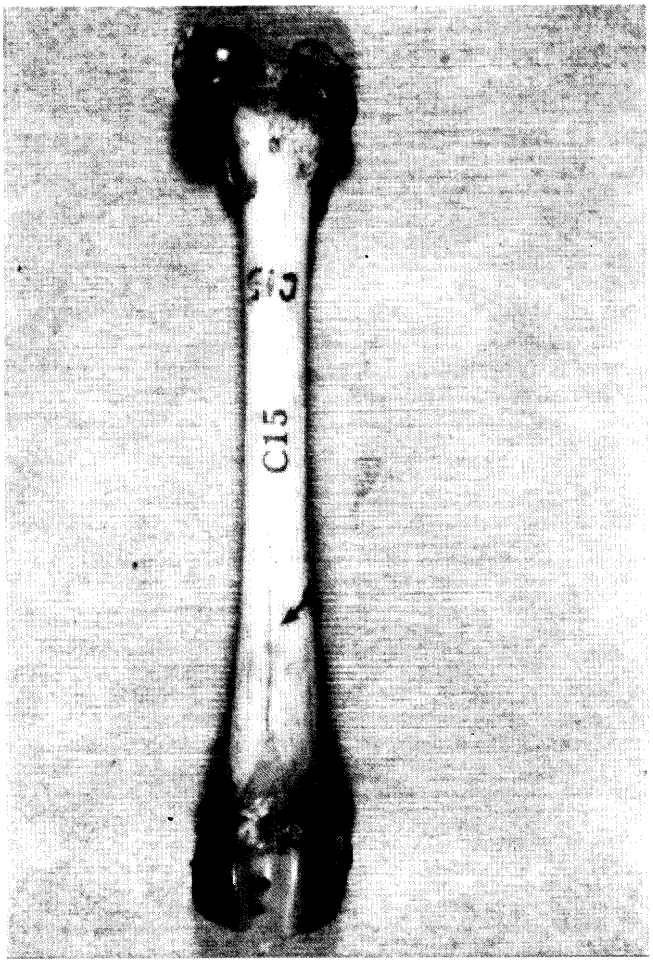


Fig 1. Longitudinal hairline cracks noted after storage at -150°C in a specimen from Group I—uncontrolled freezing and slow thawing at 1°C/min.

Discussion

Stromberg and Dalen[18] showed that canine bones stored for 48 hours at -40°C were comparatively weaker to fresh specimens by an average difference of -4.6%. Sedlin et al,[16] Pelkar et al[14,15] and Goh et al[13] were in counter-agreement and that freezing between -10°C and -60°C did not alter the torsion properties of the bone significantly. However, these were all on small animals. In our series storage at ultra low temperatures in a larger animal model showed no difference in the torsion prop-

TABLE IV: EXPERIMENTAL RESULTS OF THE PAIRED-GROUPS WHERE NO CORTICAL HAIRLINE CRACKS WERE OBSERVED PRIOR TO TESTING ARE PRESENTED AS MEAN (SD) OF THE RATIO

Paired-Group	n, pairs	Max. torque (to failure)	Max. angular rotation (to failure)	Elastic torsion stiffness
IIB : IIA	5	1.18 (0.19)	1.00 (0.21)	0.93 (0.14)
IIIB : IIIA	4	1.02 (0.3 1)	0.97 (0.43)	1.07 (0.27)
IVB : IVA	2	1.13 (0.03)	1.21 (0.41)	0.95 (0.23)

Statistical significance was tested between the pairs. No results were recorded for Group I as at least one of the pairs had a cortical hairline crack as a result of the storage process.

TABLE V: MEAN (SD) POLAR AREA MOMENT OF INERTIA (I) AND THE MEAN (SD) MAXIMUM TORSION STRESS (τ) OF THE VARIOUS GROUPS COMPARED INDEPENDENTLY

	n, pairs	Mean polar area moment of inertia, I ($\times 10^{-9}$ m^4)	Mean maximum torsion stress, τ (MPa)
Storage at - 70°C			
Uncontrolled freeze, thaw at 1°C/min	5	3.8 (1.3)	40.6 (15.0)
Freeze at 1 °C/min, thaw at 1°C/min	5	3.8 (1.1)	47.3 (13.7)
Uncontrolled freeze, Uncontrolled thaw	4	3.6 (0.9)	36.3 (9.1)
Storage at -150°C			
Uncontrolled freeze, thaw at 1°C/min	5	4.1 (1.7)	45.6 (9.1)
Freeze at 1 °C/min, thaw at 1°C/min	4	4.2 (0.6)	44.6 (8.3)
Uncontrolled freeze, thaw at 2°C/min	3	4.2 (0.4)	50.6 (15.8)

One-way analysis of variance for both the moment of inertia, I and maximum torsion stress, τ showed that no two groups were significantly different ($a = 0.05$).

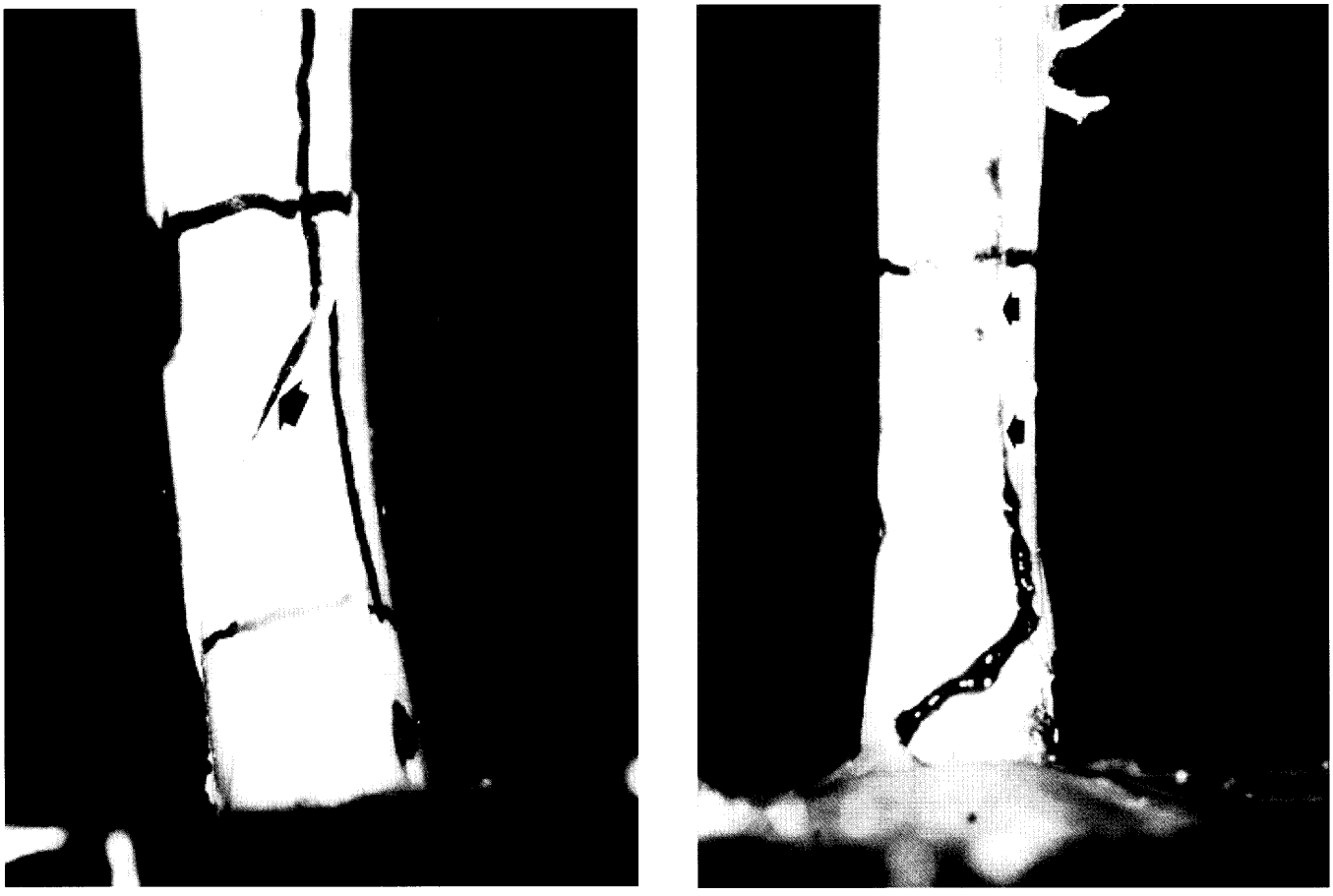


Fig. 2. Torsion test A) Bones without any longitudinal hairline cracks failed with a spiral fracture. B) Bones which had longitudinal hairline cracks as a result of the storage process failed with longitudinal fractures

TABLE VI: COMPARISON OF MEAN (SD) POLAR AREA MOMENT OF INERTIA (I) AND THE MEAN (SD) MAXIMUM TORSION STRESS (τ) OF TESTED SPECIMENS WITH AND WITHOUT CORTICAL HAIRLINE CRACK AS A RESULT OF THE STORAGE PROCESS

	n, pairs	Polar area moment of inertia, I ($\times 10^{-9}$ m^4)	Mean maximum torsion stress, τ (MPa)
Without cortical hairline cracks fractures	26	4.0 (1.1)	44.0 (11.7)
With cortical hairline cracks	10	3.8 (1.1)	22.8 (10.6)
P value		ns, $P = 0.481$	Significantly different, $P < 0.0001$

ns: not significant

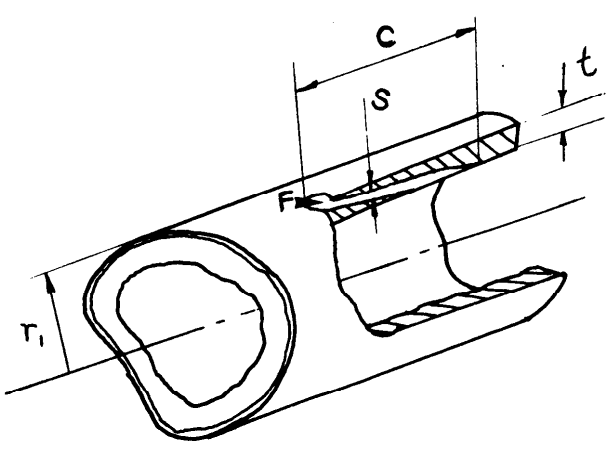
As there were no differences in the structural behaviour for the two storage temperatures, all specimens were grouped together. One-way analysis of variance for τ showed a significant difference ($\alpha = 0.05$) between the bones with cortical fracture and bones without fracture.

erties when compared to storage at -70°C.

The number of samples studied was small and this is open to criticism that there may be insufficient evidence to claim any significance. However, this study highlights the incident of cortical hairline cracks occurring as a result of the storage of whole intact bones at -70°C and -150°C, which is of clinical relevance. In this study, the occurrence of longitudinal hairline cracks is suggested to be an effect of the freezing / thawing cycle as they were not present before the process. Similar microcracks were observed by Pelkar et al in their freeze-dried specimens during rehydration.[14]

In this study, prior to freezing, both the controls and observed groups were carefully examined visually and with radiographs. The cracks seen in such cases after thawing seem to initiate at the nutrient artery foramen into the intra-medullary canal. Gross cross-sections of the diaphyseal bone at the nutrient vessel cavity showed that the cavity travelled between 5-mm to 15-mm obliquely from the cortical surface before inserting into the medullary canal (Fig. 3A). The diameter of the cavity was about 40% to 55% of the cortical thickness (Fig. 3B). This creates a stress riser in the cortex. The cracks from the cross-sectional view seem to radiate from the foramen involving the full thickness of the cortex.

There may be a possible influence of thermal stress of the bone when freezing and thawing at controlled and



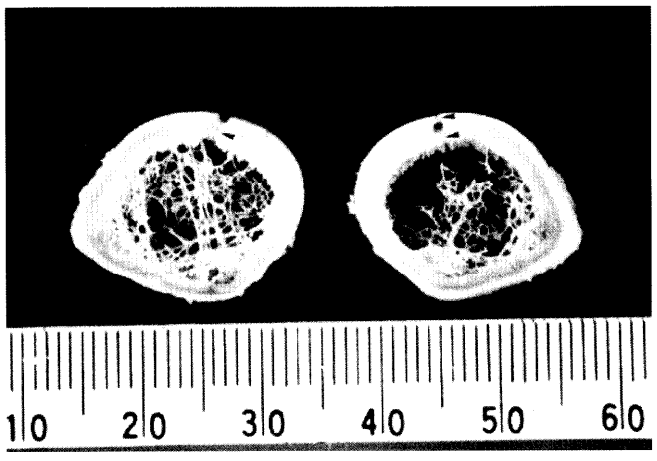


Fig. 3. A) Schematic longitudinal sections of the diaphyseal hone of outer (endosteal) radius, r_i and cortical thickness, t. The mean size of the vessel foramen, s was found to be 40-55% of thickness, t, and the mean length of the foramen, c along the cortex was found to be 10-15mm. F = nutrient vessel foramen. B) Two adjacent cross-sections of a diaphyseal bone after storage, which were observed to have a longitudinal crack. It demonstrates the size of the nutrient vessel foramen with a longitudinal hairline crack (arrow) radiating from the vessel through the thickness of the bone

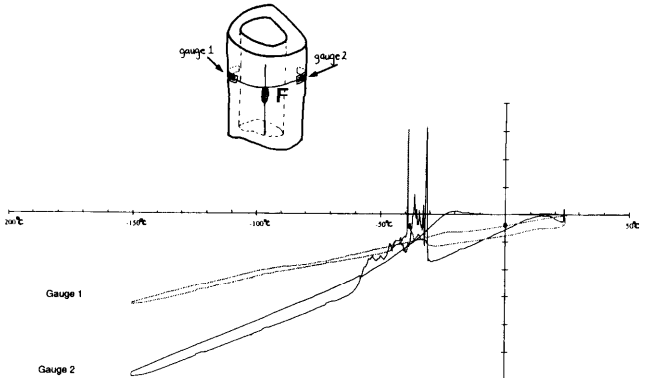


Fig. 4. Strain-temperature history curves measured during the freezing and thawing (at 1°C/min) process of the allografts (Group IB). The strain gauges 1 and 2 were placed circumferentially and perpendicular to the long axis oftheshaft. This was perpendicular to the proximal nutrient vessel foramen (F) on the bone cortex, as shown in the inserted schematic diagram, to study the effect on the tip of the so-called "deject". The strain history with temperature showed a steady gradient. During the thawing phase, at about -60°C, the strain-temperature gradient changed steeply with a sudden jump between -40°C and -30°C, returning to its previous strain rate thereafter

uncontrolled rates. However, this was assumed to be minimal. At what point in the freeze-storage-thaw cycle these hairline cracks occurred is still speculation. In a subsequent pilot study to confirm this, Group I was repeated for two specimens. Cryo-strain gauges (CFLA-1, TML, Tokyo Sokki Kenkyojo, Japan) designed for working at temperatures down to -198°C were placed orthogonally on the bone in line with the longer axis of the foramen of the more proximal nutrient vessel. The gauges were to monitor the strain during the freezing and thawing-process. The time series data seem the suggest that a change in strain-temperature rate occurred during the thawing process (1°C/min) at a temperature between -60°C and -30°C with a more abrupt change in strain between -40°C and -30°C. This change in strain quickly regained its original strain-temperature gradient (Fig. 4) suggesting a sudden expansion of the local region at this temperature. Manzur's review of the destructive effects of the rate of cooling and thawing on cells and organisms can shed some light on this occurrence.[19] He cautions that if optimum rates were not selected, the freezing of cells could be destructive especially if we do not understand the permeability of cells to water. Rapid cooling to very low temperatures can produce small ice crystals that form within rapidly cooled cells. These small ice crystals with high surface energies are likely to enlarge during thawing (re-crystallisation or grain growth), especially if the rate of thawing is slow. If cooling is slow or if the permeability of cells to water is high, equilibrium will be reached by transferring intracellular water to the external ice thus dehydrating the cells (or a solution effect). This will affect the cell volume, the water content of the cells and the molecular weight thereby affecting the cells structural properties.[5,17] With the foramen as a potential stress riser and with the intramedullary canal behaving like a closed vessel, the effects of freezing and thawing on ice crystals could pay for a fracture to propagate.

The unpredictable occurrence of longitudinal hairline cracks within the different experimental groups cannot be accredited only to the rate of thawing or cooling or the permeability of the cells at low temperatures. Other factors could be responsible, such as the size of the nutrient vessel foramen, cortical thickness, external diameter of the shaft, age, bone density or bone porosity.

It was noted that the mode of failure for the "damaged" specimens, when tested to failure, was non-spiral with the crack propagating from the hairline cracks (Fig. 2B). With external rotation applied, a vertical shift was observed along the fracture line, crack initiation and then catastrophic failure. This was unlike the "undamaged" specimens, where catastrophic spiral fractures were distinctly noted (Fig. 2A). A comparison of the maximum torsion to failure between specimens with longitudinal pre-test cracks and those without cracks

(Table VI) showed a difference of about 48% ($P < 0.0001$) suggesting that the cortical fractures weaken the bones considerably.

The occurrence of pre-test fractures seems to have been solved when the medullary canal was decompressed by drilling a cavity at the articular surface of the distal femur. This was significant for bones stored at -70°C. This result appears to suggest that the pressure within the medullary cavity might play a part in considering the occurrence of these fractures during the freezing and thawing process. As mentioned earlier, a combination of a freeze and slow thaw rate will result in recrystallisation of ice crystals, increasing in its volume. Creating a vent by drilling a hole would allow the marrow canal contents to be released, reducing the pressure in the cavity and within the nutrient artery foramen (stress risers) to sub-threshold levels, thus avoiding the occurrence of fractures. However, more work is needed to substantiate this.

The clinical relevance of this study is significant. Firstly, it may explain the high incidence of graft fractures in limb salvage surgery using frozen allografts.[4] The femur is commonly harvested whole and often does not undergo sectioning prior to freezing. Cracks that occur may go unnoticed when implanted, giving rise to graft fractures when loaded. The torsion strength of bones with cortical cracks was noted to have reduced by almost 50% even without the drilling of screw holes for plate osteosynthesis as performed clinically between host and allograft. The use of interlocking nails with and without methyl methacrylate cement may not reduce the incidence of graft fractures as these adjuncts are themselves weak in torsion resistance. Finally, the host almost never remodels allograft bone although subperiosteally, new bone may produce a form of "involucrum", thereby protecting the allograft from failure.[20]

In summary, the torsion strength of bone stored in a -70°C mechanical freezer and at -150°C in the liquid phase of nitrogen is not significantly different. However, storing intact bones at -70°C or -150°C has an unpredictable occurrence of cortical hairline cracks, especially if it is cooled to the storage temperature and slow thawed to room temperature at 1°C/min. When pre-test fractures were absent, the torsion strength was found not to be significantly different between storage at -70°C and -150°C. The hairline cracks which do occur during the storage process are dependent on the rate of freezing and thawing used, and the geometry of the bone. This can be avoided if the medullary canal is decompressed. In clinical practice, decompression of the intramedullary contents of frozen femoral allografts before storage may reduce or prevent the occurrence of stress fractures. This can be done by either sectioning at the diaphysis, through intramedullary reaming or by a washout from a vent in the intercondylar notch of sufficient diameter.

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