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Cryopreservation of osteo-chondral grafts in rabbits

To study the cryopreservation of osteoarticular allografts, a lateral femoral condyle of the rabbit was transplanted fresh, after uncontrolled freezing to –80°C with 4 weeks preservation, and after freezing 1°C per min to –100°C in 10 per cent dimethylsulphoxide medium with 4-week storage in liquid nitrogen. Autografts were used as controls.

After 3 months, the incorporation of the grafted bone was good in all technically successful cases. The $NADH_2$ diaphorase activity and ^{35}S sulphate uptake indicated well-functioning chondrocytes in all autografts. In the allografts, areas lacking enzyme activity and lacking ^{35}S uptake were observed in cartilages with otherwise normally functioning chondrocytes. No differences were found between the three allograft groups.

We conclude that freezing permits reasonably good short-term bank preservation of cartilage. We found no difference between conventional freezing and controlled slow freezing with preservative.

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Frozen osteoarticular transplants have been found useful in allotransplantation after large resections (Koskinen et al. 1979). While the incorporation of the osseous components is a rule (Salenius et al. 1982), the viability of the articular cartilage is the limiting factor which determines the long-term result after such transplantations (Parrish 1973). Freezing still appears to be the best clinically applicable method to reduce the immunogenicity of large non-vascular allogeneic bone grafts (Burwell 1963, Langer et al. 1975), but the viability and longevity of articular cartilage after clinical cryopreservation leave much to be desired.

We have compared cartilage survival in controlled deep-freezing with fresh allografting and autografting of the femoral condyle in the rabbit.

Material and methods

Thirty-two rabbits supplied by Statens Institutt for Folkehelse, Norway, and weighing between 3.0 and 4.5 kg, were divided into four groups.

Approximately two-thirds of the right lateral femoral condyle was osteotomized sagittally and a screw hole was made transversely for later osteosynthesis using Hypnorm (Leo, Sweden) i.m. and Xylocain (Astra, Sweden) as local anaesthetic. At this stage, either an orthotopic autograft transplantation was done or the condyle was replaced with an allograft.

The animals were kept in cages individually and allowed free access to food and water. Weight-bearing was not restricted, and the rabbits were allowed to move about outside their cages for short periods. All the animals gained weight normally. After 3 months, the animals were killed and specimens were submitted for morphological and histochemical studies. For autoradiography, the rabbits were given ^{35}S-sodium sulphate 1 mCi/kg i.v. 2 h before killing (Amprino 1956).

Group 1. Orthotopic autograft transplantation. In eight rabbits autotransplants were resected and immediately reinserted.

Group 2. Fresh allograft transplantation. Eight animals were operated on in pairs, each pair of animals exchanging condyles on the operating table.

Group 3. Frozen allograft after uncontrolled freezing. The allograft was stored for 2 days in a temperature of –30°C and then moved to a temperature of –80°C. Prior to transplantation 4 weeks later, the allograft was thawed in 37°C Ringer solution for 5–10 min.

Group 4. Frozen allograft after controlled freezing. The allograft was placed in a sterile plastic container, which for this group contained a sterile mixture of 1 ml 20% DMSO (dimethyl sulphoxide, E. Merck,

GFR) and 1 ml of Dulbecco's modification of Eagle's medium (Flow Laboratories, England). The containers were placed in a Controlled Rate Freezer (CRF-2, Union Carbide, England) and subjected to controlled speed freezing, 1°C/min from 20°C to −100°C. After they had reached −100°C, the tubes were plunged into liquid nitrogen (−196°C) and stored for 4 weeks. At the end of this period, and prior to transplantation, the tubes were placed in 37°C saline for 10 min and rinsed.

For *macroscopical classification* of the transplants, the following parameters were used: technical result, incorporation of the graft bone, soft tissue reaction, pannus and the state of graft cartilage.

Histological studies. Biopsies were taken systematically from six locations in the grafted cartilage and from the medial condyle for comparison. The tissue samples were processed for haematoxylin and eosin staining. Several 3-μm-thick sections, representing the whole depth of the biopsy site, were cut.

Enzyme histochemistry. The tissue specimens were immediately frozen in an isopentane container in liquid nitrogen (−140°C). Sections of 7 μm thickness were cut in a cryostat at −20°C. They were then incubated at 37°C for 30 min to show the activity of $NADH_2$ diaphorase (Chayen et al. 1973). Control incubations were carried out, omitting the substrate from the medium. Further, sections treated in distilled water at 90°C for 10 min were incubated in the complete medium. Areas without $NADH_2$ diaphorase staining of the cells were called focal necrosis, and the positive areas were graded 1, 2 or 3 according to the staining activity.

Autoradiography. Deparaffinized and rehydrated sections on slides were prepared in a dark room by the dipping procedure, using Kodak K5 nuclear emulsion. The emulsion was dissolved in distilled water at 45°C for 30 min before use and tested for background activity before application. The exposure time was 28 days in the dark at 4°C. The autoradiograms were developed in Kodak D_{19} developer at 16°C for 7 min, fixed in Kodak Rapid Fixer at room temperature for 5 min and washed 5 times for 3 min in distilled water. The sections were then stained with haematoxylin through the emulsion.

Areas having no cellular or pericellular labelling of ^{35}S compared to the background were classified as negative areas; positive areas were graded 1, 2 or 3 according to the number of silver grains.

The histological, enzyme histochemical, and autoradiographic specimens were analyzed blindly without knowing the grouping of the specimens and finally compared with the macroscopical results of transplantation.

In the *statistical analysis*, the Fisher exact probability test was used.

Results

In Groups 2 and 3, one experiment in each group was excluded due to technical failure. In all the other experiments, the macroscopic bone incorporation was good.

Table 1. Macroscopic appearance of cartilage, $NADH_2$ diaphorase activity and ^{35}S-sulphate uptake after femoral condylar transplantation

Group	Macroscopy	$NADH_2$d		^{35}S	
		Uptake	Focal necroses	Uptake	Negative areas
1					
Autografts	G	3	−	3	−
	P	3	−	3	−
	P	3	−	3	−
	G	3	−	3	−
	G	3	−	3	−
	G	3	−	3	−
	G	3	−	2	−
	G	3	−	3	−
					−
2					
Fresh allografts	P	2	+	2	−
	G	3	−	2	−
	G	3	−	2	−
	G	3	−	2	−
	P	2	+	3	−
	P	2	+	3	+
	P	3	+	2	−
3					
Uncontrolled freezing	P	3	−	2	−
	G	2	−	2	+
	P	3	−	2	−
	G	3	−	3	−
	G	2	+	3	+
	G	3	−	1	+
	G	3	−	2	+
4					
Controlled freezing	G	2	+	2	+
	P	3	+	1	+
	P	2	−	3	−
	P	1	+	1	+
	G	2	−	2	−
	G	2	−	1	+
	P	2	−	3	+
	G	3	+	3	+

G = good, P = poor. $NADH_2$d activity and ^{35}S uptake graded 1–3.

General morphology of the transplants (Table 1)

Group 1: Six results were rated as good and two as poor due to pannus formation. In five cases the cartilage was normal macroscopically and in the haemotoxylin and eosin sections. In one case, pannus formation was present, covering large areas of the cartilage surface. Microscopically the cartilage was normal. In both poor cases the cartilage was dull and eroded and the periarticular soft tissue reaction was extensive. Even in these macroscopically poor cases, histological examination showed viable cartilage with no areas of necrosis, strong staining for $NADH_2$, and strong autoradiographic labelling around the chondrocytes.

Group 2: The four poor joints had pannus varying from minimal to marked. Three had only minimal soft tissue reaction, while the fourth joint had marked reaction. On the other hand, all the joints rated as good had no or minimal soft tissue reaction and only minimal pannus formation. Areas of focal necrosis were found in all poor transplants while the medial condyles showed good staining.

Negative autoradiographic areas were found in only one of seven condyles. This condyle had a generally strong staining pattern not unlike the controls.

Group 3: One joint rated as poor had large areas of dead cartilage with no soft tissue reaction or pannus formation. Pannus and soft tissue reaction were as in Group 2. Focal necroses were found in one condyle. One specimen showed a total lack of labelling in autoradiography, while three others showed negative areas but with generally strong staining patterns comparable to controls outside these areas.

Group 4: All four joints rated as poor had clear soft tissue reaction and pannus formation, in contrast to the remaining four good joints, which also had good articular surfaces, except for one with a limited erosion. Focal necroses were found in one-half of the condyles (Figures 1 and 2). Negative autoradiographic areas were found in six condyles with normal staining pattern outside these areas (Figures 3 and 4).

No significant differences were found between the macroscopic or microscopic morphological results of auto- and allotransplantations. When the results of enzyme histochemistry in all groups were compared concerning the presence of focal necroses, the autogeneic transplantation group, which did not contain any specimens with focal necrosis, differed ($P = 0.026$) from the fresh allografts. Also, when the autografts were compared with all allografts, a difference ($P = 0.035$) was demonstrated.

The autogenetic transplants did not contain any areas without uptake of ^{35}S. Tested against all allografts the result was better ($P = 0.007$) in the autogeneic transplantation group. When the various transplantation groups were compared concerning the macroscopic, histologic and autoradiographic picture, the following as-

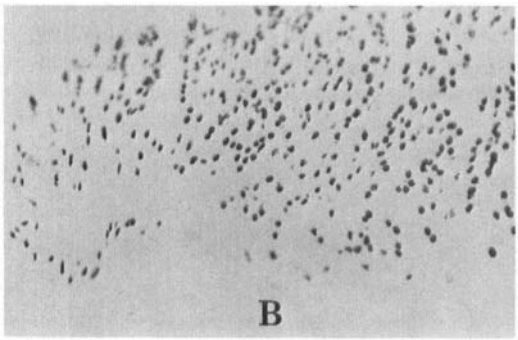

Figure 1. Normal cartilage showing $NADH_2$ diaphorase staining of the cells. B = bone. Group 4. (× 112).

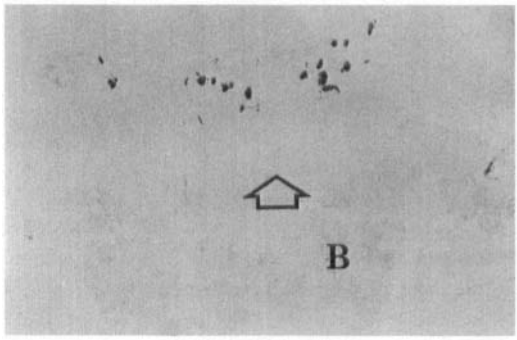

Figure 2. Cartilage with only a few $NADH_2$ diaphorase positive cells. Note the unstained necrotic area (arrow). B = bone. Group 4. (× 112).

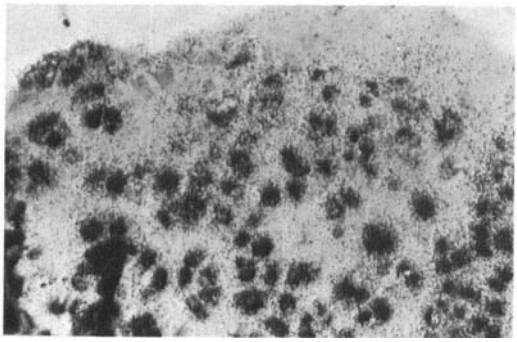

Figure 3. Cartilage with autoradiographically $^{35}SO_4$ labelled cells. Group 4. (× 280).

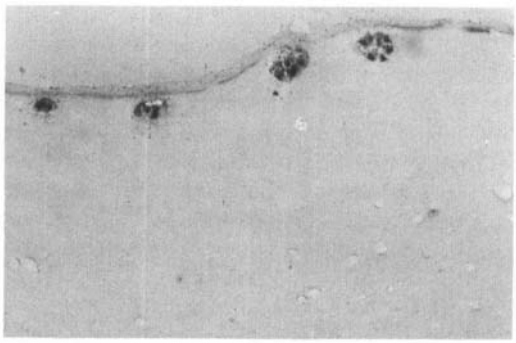

Figure 4. Cartilage showing areas without ^{35}S-sulphate deposits, which demonstrates defective synthesis of extracellular matrix. Group 4. (× 112).

sociations were revealed. In 15 cases with good macroscopic results and six cases with poor results, no focal necrotic areas were found in the enzyme histochemistry studies. Focal necroses were found in three cases with good result and in six cases with poor result. Thus, a good macroscopic result was ($P = 0.031$) associated with lack of focal necrosis. There was also an association ($P = 0.053$) between the enzyme histochemistry and autoradiography studies. In 16 cases neither focal necrotic nor negative uptake areas were observed. In eight cases, a discrepancy was observed between focal necrosis and lack of uptake with the presence of one but not the other. In six specimens focal necrotic areas and negative uptake areas were observed simultaneously. No correlation was found between the macroscopic results and the uptake distribution of ^{35}S.

The control samples taken from the medial femoral condyles of the 30 successful cases all showed normal morphology and function (grade 3, Table 1). In the subchondral bone, vascularization was proceeding normally and no collapsed, destroyed bone was observed.

Discussion

Our osteoarticular transplant model, the lateral femoral condyle, which we have not seen reported before, functioned mechanically well in 30 of 32 cases. The joint congruity was good, and the creeping substitution so far advanced that the transplant was solidly united at 3 months, confirming earlier experimental and clinical findings concerning the incorporation and integrity of the osseous component (Burwell 1963, Langer et al. 1975, Koskinen et al. 1979, Mankin et al. 1982, Salenius et al. 1982).

During freezing and thawing, chondrocytes in articular cartilage suffer various grades of damage and are not replaced, unlike osteoid-matrix-forming cells. This has consequences for the quality of the cartilage. Since slow, controlled freezing improves cell preservation (Farrant 1980, Tomford et al. 1982), we were interested in comparing speed-controlled freezing and dimethylsulphoxide preservation with the uncontrolled, stepwise clinical freezing method and fresh allografting. ^{35}S sulphate was used as an indicator of the proteoglycan synthetic activity of the chondrocytes (Amprino 1956). $NADH_2$ diaphorase, which reflects the microsomal respiratory enzyme activity, was chosen as a general indicator of the integrity of cell function (Chayen et al. 1973).

Our macroscopic evaluation of the state of the cartilage was not very reliable. Adhesions, pannus and dull surface of the cartilage were not necessarily indicators of non-vital cartilage. On the other hand, microscopic focal necroses were more often present in the poor allograft cases where macroscopic necrotic areas were also present. A poor macroscopic appearance of the cartilage may, of course, be a primary stage in the process of cartilage degeneration.

Although our methods were not quantitative, it can be concluded that large parts of cartilage had retained their metabolic activity. The deterioration of the frozen and thawed cartilage was patchy as judged by both histochem-

istry and autoradiography. Our results were very different from those of Simon and associates (1976) who used *in situ* freezing of articular cartilage as a model: from 2 to 6 months they did not observe any significant uptake of ^{35}S. They concluded that the freezing had resulted in death of the cartilage cells. This appears to support slow controlled freezing as used in our study. Our findings were similar in fresh and frozen-thawed allograft transplants, and it may be that a longer observation period would have differentiated better between the groups.

We conclude that osteoarticular condylar allografts incorporate well by creeping substitution, and that the fate of cartilage is the limiting factor. It appears that freezing preservation, which may be immunologically preferable and allows banking, in clinical allografting practice leaves functioning chondrocytes. Special methods have to be developed for slow, regulated freezing of large clinical allografts.

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