Cryopreserved Porcine Tendons Preserve Cell Viability After Thawing


ABSTRACT

Controlled cryopreservation is an important method for storage of tissue grafts in skin banking, reproductive medicine and other domains. Although the availability of cryopreserved flexor tendons would be highly beneficial in reconstructive surgery, especially in complex reconstructions for which grafting material is limited, only a few studies have dealt with transplanted tendons. We achieved successful cryopreservation of porcine flexor tendons in 2 cryoprotective media: dimethyl sulfoxide and glycerol. Their viability was shown using a quantitative colorimetric MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay. For comparison of native and cryopreserved tendons (n = 7 samples each), the adopted viability index was the ratio of MTT-dependent optical density and tendon weight. The viability index of native samples did not change significantly after cryopreservation and thawing. The proliferative capacity of tendon fibroblasts after thawing was shown in primary cell cultures. The described cryopreservation protocol and MTT assay may provide a basis for future autografting of human tendons.

TENDON DAMAGE as a result of trauma and injuries demands reconstructive surgery. In case of irreversible tendon damage, tendon autografting is the first choice. Autografts are superior to allografts or xenografts because of the lower failure rate and the prevention of an immune reaction.1–3 The limited availability of tendon grafts could benefit from preservation of tissue obtained from injured or amputated limbs from multiply traumatized patients. Cryopreservation could provide tendon grafts for multiple-stage reconstructions.

In the present study, we used a standardized cryopreservation protocol for tendon storage. We showed the applicability of the quantitative colorimetric MTT assay for split-skin grafts4–6 to monitor tendon viability after thawing. The viability assay uses the yellow tetrazolium compound MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) which is reduced by mitochondrial enzymes of living cells to purple formazan, which is assessed colorimetrically.7 We demonstrated high viability of cryopreserved compared with native tendon samples of pig flexor tendons using dimethyl sulfoxide (DMSO) and glycerol as cryoprotective agents.8 Furthermore, we observed that the MTT-assay was suitable to monitor the viability of tendon grafts for surgical application.

MATERIALS AND METHODS

Isolation of Tendon Samples

Tendon samples were obtained from the feet of adult pigs at the local abattoir. The forefeet were ablated immediately after death and transported on ice to the laboratory. The tendons of the musculus flexor digitorum profundus were dissected under sterile conditions, pulled out from the tendon sheath, and stored in ice-cold 0.01M phosphate-buffered saline solution. Samples were available for further processing within 5 hours of sacrifice.

Cryopreservation

The cryopreservation technique was based on the reports of split-skin grafts.5 The cryoprotective medium Dulbecco modified Engle medium (D-MEM)/Ham F-12 (Gibco BRL, In vitro Corp, Carlsbad, California) contained 10% DMSO or 10% glycerol. Tendon samples were cryopreserved in either cryoprotectant (n = 7 donor animals in each group). Three tendon samples at a time were sealed in a 17.5 × 22-cm sterile aluminum pouch (MacoPharma SA, Tourcoing, Nord, France) loosely floating in 10 mL of the medium. For optimal penetration, samples in glycerol were incubated at room temperature for 40 minutes, and those in DMSO at 4°C for 20 minutes. Subsequently, the pouches placed in an 18 × 22-cm aluminum box were inserted in a controlled-rate freezing chamber (Kryo-10; Messer Griesheim GmbH, Frankfurt, Germany). The temperature was downregulated at 2°C/min to −120°C. The pouches were transferred in the vapor phase of a
Viability Testing

The viability of the tendon samples was assessed by their ability to reduce MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich, St. Louis, Missouri) to purple DMSO-soluble formazan. The samples were obtained by punch biopsy (Ø 4 mm) of manually sliced tendon disks, discarding the epitenon. Uniform sample size was important to achieve equal concentration of the MTT-salt within the probe.

To establish a quantitative MTT assay, parameters of incubation time and dry tissue weight of native tendon samples were assessed. To analyze the relationship between the weight of the probes and the optical density (OD) of the produced formazan, various numbers of the probes per well were cultured in 24-well tissue culture plates in 400 µL of D-MEM/Ham F-12 medium which had been preheated for 30 minutes at 37°C in a humidified atmosphere at 5% carbondioxide. Thereafter, MTT and sodium succinate (final concentrations, 0.83 mg/mL and 1.25 mg/mL) were added to the wells and incubated for an additional 3 hours. The samples were then transferred into Eppendorf tubes and submerged in 500 µL DMSO in the dark at 37°C overnight to extract the formazan deposits from the tissue. The optical density (OD) of the solvent was measured with a microplate spectrophotometer (Molecular Devices, Sunnyvale, California) at 570 nm. The tissue probes were dried at 60°C overnight in an incubator and weighted. The OD values were plotted vs dry weight (in milligrams).

For comparison of the viability of fresh and preserved tendons, 12 individual punches from 1 tendon sample per donor animal were analyzed using the described assay. Negative controls of devitalized tendon samples (fixed in 4% formaldehyde) were included in the MTT assay. The viability index (OD/mg) was calculated as the ratio of the OD and dry weight of individual samples (mg) diminished by the viability index of the devitalized probes. The viability index was always less than 10% of the native samples. Data were analyzed using commercially available software (SPSS version 5.0; SPSS, Inc, Chicago, Illinois) and are expressed as mean (SD). The individual groups were tested for comparison of the viability of fresh and preserved tendons using the unpaired t-test. Values were considered statistically significant if a P value was < 0.05.

RESULTS

MTT Assay of Native Tendon

Preliminary experiments with the native tendon samples showed that 1 to 3 hours of incubation is adequate for conversion of MTT salt to visible amounts of deep-blue formazan detected colorimetrically. Regression analysis demonstrated a positive linear correlation of sample size and OD ($R^2 = 0.97$; Fig 1). The reducing MTT activity of tendon cells was, therefore, adopted for quantification of tendon viability.

DISCUSSION

The present study demonstrates that the controlled cryopreservation of flexor tendon samples did not significantly affect tendon cell viability. The evaluation was based on the viability index (OD/mg), which was calculated using the quantitative colorimetric MTT assay and the tendon dry weight. Several studies have used the MTT assay as a prognostic parameter for cryopreserved skin grafts. We used the MTT assay for tendons although the tetrazolium-reductase activity was expected to be low. In contrast with skin, which has about 95% of cells related to the dry mass, the cellular content of tendons is only 3%. Nevertheless, small (2–4 mg) endotenon samples contain sufficient metabolically active cells to produce an OD signal of about 0.28 to 0.55 after 1 hour of incubation.

The viability index of native tendon samples determined using the MTT assay was 0.13 (0.03) OD/mg. This value was not significantly affected when tendon samples were subjected to a controlled freezing process and then thawed.
after storage for 130 days in liquid nitrogen. The performance of 2 cryoprotective media, 1 containing 10% DMSO and the other 10% glycerol, was comparable. Another study compared the number of viable cells derived from native and cryopreserved tendons after collagenase digestion and found retention of cellular viability to be 92.5%. The high survival rate was in agreement with our observation of a similar viability index before and after cryopreservation. The vitality of human split-thickness skin grafts cryopreserved for 1 to 6 months assessed using MTT showed a viability index of 60%. The viability of cryopreserved cartilage was as high as 75%. We have no explanation for why tendon cells are particularly tolerant to cryopreservation. Further studies are required to demonstrate whether tendon cells, their matrix components or the intrinsic tendon growth factors might expert protective effects. The potential of cryopreserved tendon autografts must be shown in an animal model.

Our study results demonstrate that a controlled cryopreservation process has a nonsignificant effect on the viability of native tendons. As an inexpensive, fast, and reliable prognostic parameter, the MTT assay is suitable for assessment of the viability of cryopreserved tendon autografts. Because of the growing demands for trauma surgery, tendon cryopreservation would be extremely helpful in late-management reconstruction.

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REFERENCES