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Original article

Biomechanical and immunohistochemical analysis of high hydrostatic pressure-treated Achilles tendons

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Abstract

Background. Reconstruction of bone defects caused by malignant tumors is carried out in different ways. At present, tumor-bearing bone segments are devitalized mainly by extracorporeal irradiation or autoclaving, but both methods have substantial disadvantages. In this regard, high hydrostatic pressure (HHP) treatment of the bone is a new, advancing technology that has been used in preclinical testing to inactivate normal cells and tumor cells without altering the biomechanical properties of the bone. The aim of this study was to examine the biomechanical and immunohistochemical properties of tendons after exposure to HHP and to evaluate whether preservation of the bony attachment of tendons and ligaments is possible.

Methods. For this, 19 paired Achilles tendons were harvested from both hindlimbs of 4-month-old pigs. After preparation, the cross-sectional area of each tendon was determined by magnetic resonance imaging (MRI). For each animal, one of the two tendons was taken at random and exposed to a pressure of 300 MPa ($n = 9$) or 600 MPa ($n = 10$).

Results. The contralateral tendon served as an untreated control. The biomechanical properties of the tendons remained unchanged with respect to the tested parameters: Young's modulus (MPa) and tensile strength (MPa). This finding is in line with immunohistochemical labeling results, as no difference in the labeling pattern of collagen I and versican was observed when comparing the HHP group (at 600 MPa) to the untreated control group.

Conclusions. We anticipate that during orthopedic surgery HHP can serve as a novel, promising methodical approach to inactivate Achilles tendon and bone cells without altering the biomechanical properties of the tendons. This should allow one to preserve the attachment of tendon and ligaments to the devitalized bone and to facilitate functional reconstruction.

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Introduction

Treatment of cancer patients presenting with a solid malignant tumor of the bone or soft tissue requires complete resection of the infiltrated tissue, neoadjuvant chemotherapy, and eventually consecutive reconstruction. Various treatment modalities have been established for the reconstruction of bone and joint defects, including endoprostheses,[1] artificial bone substitutes,[2] and allogeneic transplants.[3] However, each of these reconstruction methods is associated with certain limitations and complications for the patient; thus, new, innovative strategies are strongly desired.

The reconstruction of tendons is highly essential for the restoration of joint function, but the refixation of tendons and ligaments to artificial implants is a problematic issue. In this sense, biological reconstruction could help avoid such functional limitation, but allogeneic transplantation of tendons is still a matter of concern, as such a transplant may be contaminated by viruses or bacteria[4] and therefore requires special awareness of procurement, storage, and sterilization.[5]

An alternative approach for limb reconstruction of tumor-affected bone is extracorporal irradiation or autoclaving of resected tissue segments and subsequent reimplantation. For this, it is necessary to devitalize the tumor cells completely to avoid tumor recurrence. Still, with this treatment, destruction of tendons, ligaments, and bone does occur associated with severe loss of biomechanical and biological properties and is therefore a central concern in orthopedic surgery.[6–10] Consequently, it is necessary to develop an adequate procedure that would achieve tumor cell inactivation of a bone or tendon without compromising the biomechanical properties of bone, tendons, and ligaments.[6–10]

In our own recent studies, the effect of high hydrostatic pressure (HHP) on viability of various human tumor-afflicted tissues, tumor cell lines, and normal cells was investigated. At an exposure of 350 MPa

(10 min, 37°C), all normal and tumor cells were damaged and devitalized.[11–13] Also, we observed that at a high pressure of 600 MPa the biomechanical properties of bones remain unchanged.[14] Until now, the effect of HHP on the biomechanical and biological properties of tendons has not been described, although HHP — as an alternative to irradiation or autoclaving — could offer new perspectives to treat tendons ex vivo after en bloc resection, thus maintaining the natural attachment of the tendons and ligaments to the bone.

Material and methods

Tendon specimens and MRI examinations

Nineteen paired Achilles' tendons were harvested from both hindlimbs of 4-month-old pigs. The surrounding tissue was removed, and the tendons were stored in absorbent towels (Kimberly-Clark, Zaventem, Belgium) soaked with Ringer's solution at 4°C overnight. The cross-sectional area of each tendon was determined by magnetic resonance imaging (MRI). In brief, each tendon was contrast-medium-mounted in a specially designed device filled with 10 ml dye (Magnevist; Schering, Berlin, Germany) and 1000 ml Aqua dest. While measuring the cross-sectional area, a pretension by a constant load of 3 N was applied to each tendon in an axial direction.[15] The specimens were examined at 1.5 Tesla using a clinical MR scanner (ACS NT with Intera upgrade; Philips, Best, The Netherlands). Five T1-weighted images [echo time (TE) 40 ms, repetition time (TR) 580 ms] were obtained from the midsection of the tendons with a slice thickness of 3 mm and a field of view of $100 \times 100\,mm^2$. The cross-sectional area of each slice was calculated as the mathematical product of the amount of pixels representing the tendon tissue and the area of a single pixel. The definitive cross-sectional value is given as the mean value of the five measurements.

HHP treatment of tendons

For each animal ($n = 19$) one of the paired tendons was taken at random and placed in Ringer's solution and packed in plastic bags by vaccum packing, thus avoiding air bubbles. The bags were placed in the central cavity of a water-filled pressure chamber of a high-pressure device (Dunze, Hamburg, Germany) (Fig. 1). The bags were then exposed to pressures of 300 and 600 MPa, respectively. Nine specimens were exposed to a pressure value of 300 MPa, and ten specimens were HHP-treated at 600 MPa. The specimens were held under pressure for 20 min at 20°C. The compression and decompression rate was 200 MPa/min. The untreated tendons from the contralateral legs were used as control specimens for each corresponding treatment group.

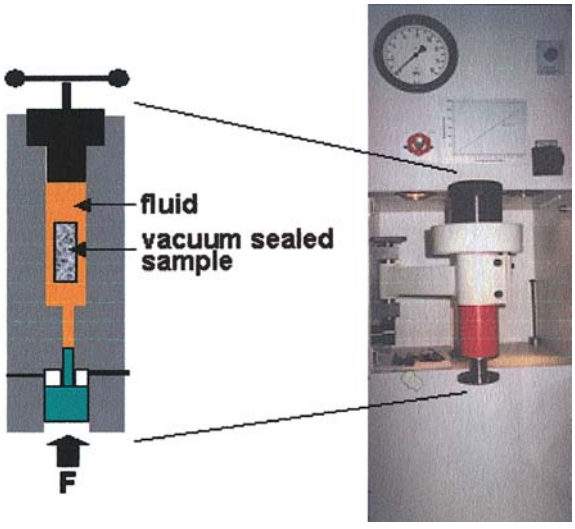


Fig. 1. High-pressure apparatus for high-pressure treatment of tendons with a drawing of the pressure chamber

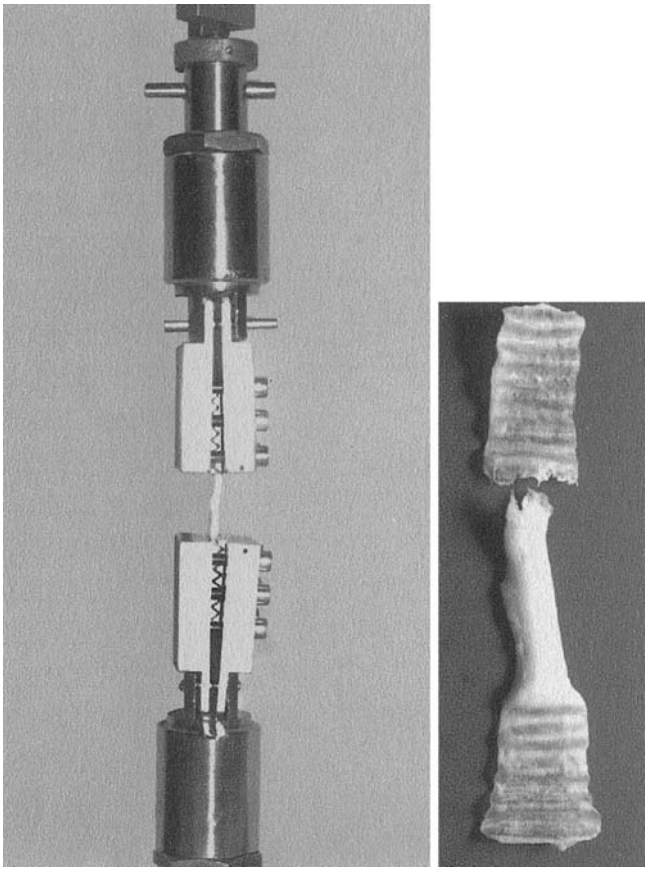
Biomechanical testing

Before and after HHP, tendons (overall length ranging from 80 to 100 mm) were exposed to a uniaxial tensile test. All of the tendons were prestrained twice up to 400 N to achieve preconditioning. The third loading cycle was performed until failure. The length of the tendons between the clamping devices was adjusted to 40 mm for all specimens for the test. Testing was performed using a universal testing machine (Zwick 1120; Zwick, Ulm, Germany) at an extension rate of 200 mm/min starting at a preload of 5 N (Fig. 2). Young's modulus (MPa) and ultimate tensile strength (MPa) were calculated by the software testXpert (release 8.1; Zwick) from the load deformation curves that were continuously recorded during testing.

Immunohistochemical staining

Details of the immunohistochemical procedures have been published previously.[16,17] Before and after HHP, Achilles tendon specimens were fixed in 90% methanol at 4°C, rinsed in phosphate-buffered saline (PBS), and then infiltrated for 2 h with 5% sucrose in PBS. Cryosections with a thickness of 12 µm were cut and immunolabeled with mouse biotin-conjugated monoclonal antibodies against type I collagen (Clone Col1; Sigma-Aldrich, Munich, Germany) or versican (Clone 12C5; Developmental Studies Hybridoma Bank, Iowa

City, IA, USA). Endogenous peroxidase activity was blocked in all sections by pretreatment with 0.3% H_2O_2 in methanol for 30 min, and nonspecific binding of the antibody was reduced with an appropriate serum block (40 min). Nonspecific binding of the secondary antibody was controlled by omitting the primary antibodies. Antibody binding was visualized using the avidin-biotin-peroxidase method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA, USA). Nuclei were counterstained with Mayer's hematoxylin in selected sections.



a,b

Fig. 2. **a** Uniaxial tensile failure test conducted on a universal testing machine. **b** Ruptured tendon after having exceeded the tensile strength

Statistics

Results of the material characteristics were expressed as median values plus the range including maximum and minimum values. Comparisons between groups were calculated by using the Wilcoxon's rank-sum test. The significance level was set at 0.05.

Results

The effect of HHP (300 or 600 MPa) on pig achilles tendons was investigated by probing possible changes in biomechanical properties after HHP treatment. There was no significant difference observed in the cross-sectional area of the tendons with a minimum of 17.9 mm^2 and maximum of 26.1 mm^2 (median of 20.1 mm^2) compared to the contralateral control between 12.1 mm^2 and 25.8 mm^2 (median of 20.0 mm^2). The mechanical properties of the pig hindlimb tendon specimens were tested in accordance with the methods described before. The material characteristic values for Young's modulus and the ultimate tensile strength of untreated control tendons and those after HHP treatment at 300 MPa ($n = 9$) or 600 MPa ($n = 10$) are listed in Table 1. No pressure-dependent significant difference was observed for the tendons treated at HHP of 300 MPa or 600 MPa, and no difference was observed compared to the untreated tendons.

The biomechanical results were in line with the results of the immunohistochemical investigation regarding collagen I and versican. Because the immunohistochemistry labeling method applied in this study

Table 1. Comparison of median levels of Young's modulus and ultimate tensile strength of pig Achilles tendons after treatment at 300 and 600 MPa compared with controls

	HHD 300 MPa ($n = 9$ per group)		HHD 600 MPa ($n = 10$ per group)	
Parameter	Treated tendons	Controls	Treated tendons	Controls
Young's modulus (MPa)				
Median	340	321	314	294
Maximum	424	386	456	409
Minimum	287	237	256	248
Ultimate tensile strength (MPa)				
Median	63	56	62	62
Maximum	68	63	80	76
Minimum	43	29	33	42

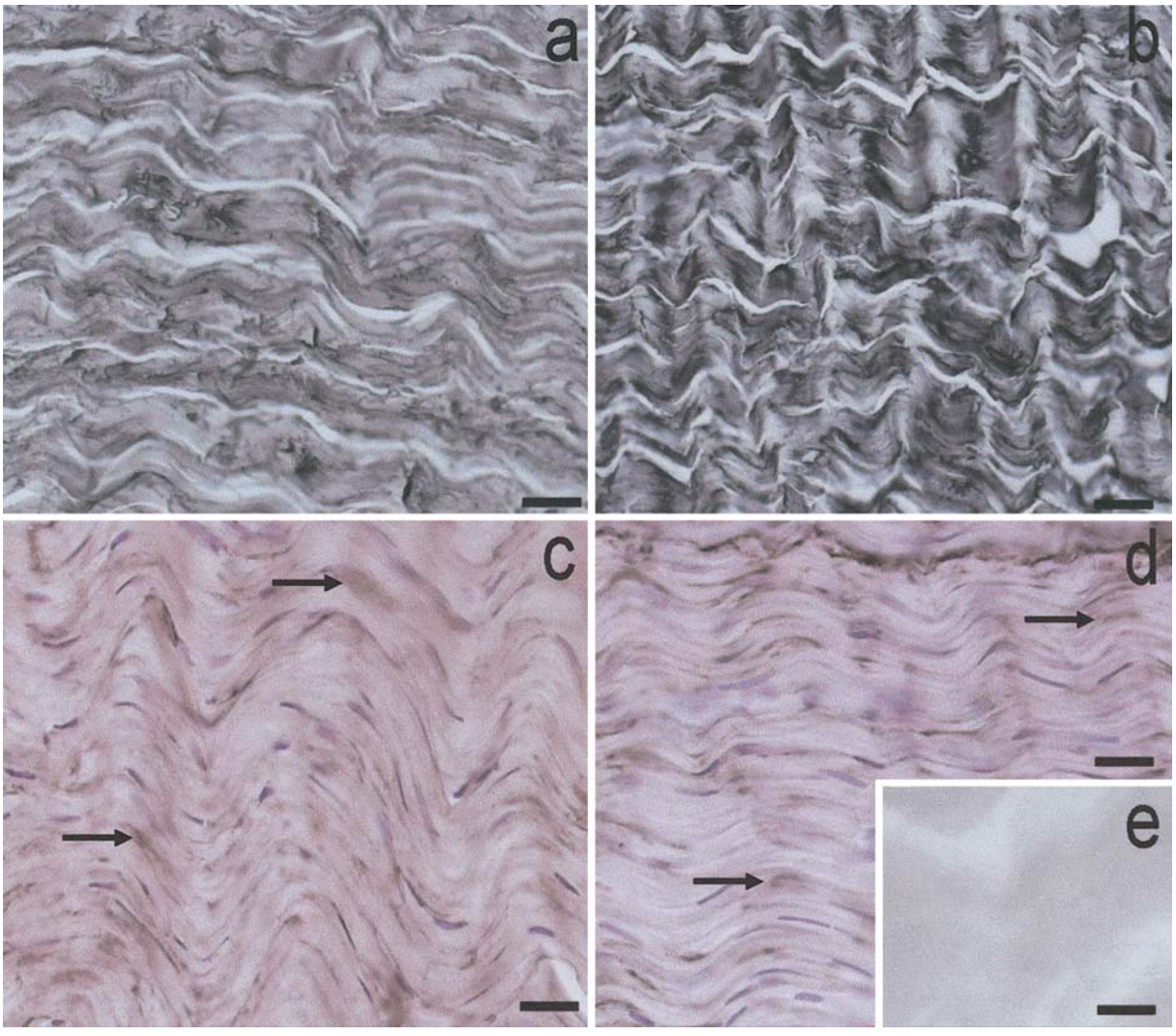


Fig. 3a–e. Immunohistochemical staining of high hydrostatic pressure (HHP)-untreated and HHP-treated tendons. Tendons were stained with monoclonal antibodies against collagen I. **a**, **b** No counterstain. *Bars* 40 µm. Note the intense black staining and versican. **c**, **d** Counterstained with Mayer's hematoxylin. *Bars* 20 µm. Note the diffuse brown staining (*arrows*). The control groups (**a**, **c**) without HHP treatment (0 MPa) exhibits a labeling pattern similar to that of the HHP-treated groups (**b**, **d**) after treatment at 600 MPa. **e** Immunohistochemical section without primary antibody and with no counterstain. *Bar* 20 µm

does not allow quantitative conclusions, only a qualitative evaluation (i.e., presence or absence of antigen ingestion) was performed. Both HHP-treated tendons and untreated control tendons showed positive reactions for type I collagen and versican. Both antigens were detected in all regions of the tendon, with no difference in the labeling pattern for 300 or 600 MPa. Representative microscopic images are shown in Fig. 3.

Discussion

This study for the first time demonstrates that excessive HHP treament is not changing the biomechanical properties of tendons, thus maintaining a good possibility for tendons and ligaments to retain their functional properties after HHP sterilization. Therefore, there is a high probability that even after HHP the mechanical properties of the bone-attached tendons or ligaments are conserved. The present results reveal that for tendons after exposure between 300 and 600 MPa the biomechanical material characteristics (Young's modulus and tensile strength) remain unchanged. This finding is supported by the still-unchanged immunohistochemical detection of type I collagen and versican in both the HHP-treated groups (at 300 and 600 MPa) and in the nonpressurized control group.

Certainly, our data provide only the first evidence of the primary stability of tendons after pressure application and give no clues concerning long-lasting tendon

strength after reimplantation. We cannot exclude the possibility that significant loss of strength of the tendon graft after HHP treatment may occur owing to necrosis and devitalization of the tendon tissue after reimplantation, a phenomenon well described for anterior cruciate ligament (ACL) transplants.[18,19] Biomechanical investigations soon after patellar tendon transplantation for ACL replacement depicted a significant decrease in graft strength during the postoperative period.[20,21] It is worth mentioning that these processes are independent of pressure application, and revascularization of the tendon grafts may lead to elevated permanent stability.[20,21]

Regarding malignant bone tumors, reimplantation of tumor-afflicted bone segments together with attached tendons after irradiation or autoclaving has several advantages over allogeneic bone transplants or prosthesis[5–8]; however, biomechanical and biological alterations due to these traditional inactivation processes limit their use.[7,22] Gamma irradiation, autoclaving, or the use of chemicals such as ethylene oxide considerably changes tissue integrity and the biomechanical stability of the bone and tendon grafts.[23–26] Therefore, it is highly desirable to establish an adequate retransplantation procedure that allows secure tumor cell inactivation while at the same time osteoconductive, osteoinductive, and biomechanical properties of the bone and tendon autograft are preserved.[9,26,27] In this regard, HHP treatment is an advancing technology, now being used in preclinical testing to inactivate tumor cells while preserving the biomechanical and biological properties of the underlying bone tissue grafts. To achieve this goal, we hypothesized that the biomechanical properties of tendons after HHP treatment would also be maintained. This would be an advantage to the patient by keeping his or her ligament attached to the then devitalized autograft, thereby allowing acceptable mechanical joint function. This is clinically relevant regarding the reconstruction of the knee extensor mechanism e.g., when the tumor involves the patellar ligament, or for reconstruction of complicated joints such as the elbow, wrist, or ankle, where preserving ligaments and tendons is a fundamental issue for their functionality.[28] Apart from the tendon transplants, we have reported recently on unchanged biomechanical properties of freshly resected human cortical and trabecular bone specimens after HHP treatment with HHP as high as 600 MPa.[14] Moreover, drastic changes in the biological properties of the extracellular matrix proteins fibronectin, vitronectin, and collagen-I were not observed under these treatment conditions.[29]

The biomechanical and biological advantages of HHP over autoclaving or irradiation are based on the fact that HHP exerts no negative effects on covalent molecular bonds. For instance, natural compounds such as flavors and aromas, as well as extracellular matrix proteins and pharmacologically active molecules, are not destroyed by HHP, and one may speculate that the structural proteins of the tendon grafts are not affected, which remains to be elucidated.[30] Cells, on the other hand, are destroyed by HHP, leading to irreversible inactivation and devitalization of bacteria and eukaryotic cells including tumor cells, as our recent investigations have demonstrated that tumor cells in human tumor-afflicted bone specimens were irreversible damaged after HHP treatment at pressure levels of 300 MPa.[31]

Conclusions

We conclude that although tumor cells can be irreversibly damaged by excessive HHP,[11–13] the structural components of bone,[14] cartilage,[32] and tendons remain unchanged, even at exposures as high as 600 MPa. Therefore, one has to regard HHP as a novel methodical approach during orthopedic surgery for tumor cell inactivation of osteochondral segments also targeting the attached tendons and ligaments, with the advantage of a functional reconstruction. Nonetheless, before clinical testing, further ex vivo and animal studies are required to demonstrate, that extracorporeal HHP eradicates tumor cells prior to retransplantation.

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References

1. Morris HG, Capanna R, Del Ben M, Campanacci D. Prosthetic reconstruction of the proximal femur after resection for bone tumors. J Arthroplasty 1995;10:293–9.
2. Mittelmeier H, Mittelmeier W, Gleitz M. Pyrost, a spongious, mineral bone substitute: experimental basics and 13 years of clinical experience in more than 1000 cases. Orthopade 1998;27:126–35 (in German).
3. Enneking WF, Campanacci DA. Retrieved human allografts: a clinicopathological study. J Bone Joint Surg Am 2001;83:971–86.
4. Knaepler H, Ascherl R, von Garrel T, Gotzen L. Guidelines for allogeneic bone transplantation. Chirurg 1991;62:763–5 (in German).
5. Sanjay BK, Moreau PG, Younge DA. Reimplantation of autoclaved tumour bone in limb salvage surgery. Int Orthop 1997;21:291–7.
6. Ahmed AR, Watanabe H, Takagishi K. Reconstruction with autologous pasteurized whole knee joint. I. Experimental study in a rabbit model. J Orthop Sci 2003;8:170–9.

7. Böhm P, Springfeld R, Springer H. Re-implantation of autoclaved bone segments in musculoskeletal tumor surgery: clinical experience in 9 patients followed for 1.1–8.4 years and review of the literature. Arch Orthop Trauma Surg 1998;118:57–65.
8. Böhm P, Fritz J, Thiede S, Budach W. Reimplantation of extracorporeal irradiated bone segments in musculoskeletal tumor surgery: clinical experience in eight patients and review of the literature. Langenbecks Arch Surg 2003;387:355–65.
9. Urist MR, Hernandez A. Excitation transfer in bone: deleterious effects of cobalt 60 radiation-sterilization of bank bone. Arch Surg 1974;109:486–93.
10. Yamamoto N, Tsuchiya H, Nojima T, Sumiya H, Tomita K. Histological and radiological analysis of autoclaved bone 2 years after extirpation. J Orthop Sci 2003;8:16–9.
11. Diehl P, Schmitt M, Blumelhuber G, Frey B, van Laak S, Fischer S, et al. Induction of tumor cell death by high hydrostatic pressure as a novel supporting technique in orthopedic surgery. Oncol Rep 2003;10:1851–5.
12. Diehl P, Reuning U, Gollwitzer H, Magdolen U, Gerdesmeyer L, Schauwecker J, et al. Effect of extracorporal high hydrostatic pressure on tumor cell adherence and viability. Oncol Rep 2004; 12:369–73.
13. Naal FD, Mengele K, Schauwecker J, Gollwitzer H, Gerdesmeyer L, Reuning U, et al. High hydrostatic pressure-induced cell death in human chondrocytes and chondrosarcoma cells. Anticancer Res 2005;25:1977–82.
14. Steinhauser E, Diehl P, Hadaller M, Schauwecker J, Busch R, Gradinger R, et al. Biomechanical investigation of the effect of high hydrostatic pressure treatment on the mechanical properties of human bone. J Biomed Mater Res B Appl Biomater 2005;76: 130–5.
15. Noguchi M, Kitaura T, Ikoma K, Kusaka Y. A method of in-vitro measurement of the cross-sectional area of soft tissues, using ultrasonography. J Orthop Sci 2002;7:247–51.
16. Milz S, McNeilly C, Putz R, Ralphs JR, Benjamin M. Fibrocartilages in the extensor tendons of the interphalangeal joints of human toes. Anat Rec 1998;252:264–70.
17. Milz S, Regner F, Putz R, Benjamin M. Expression of a wide range of extracellular matrix molecules in the tendon and trochlea of the human superior oblique muscle. Invest Ophthalmol Vis Sci 2002;43:1330–4.
18. Butler DL, Grood ES, Noyes FR, Olmstead ML, Hohn RB, Arnoczky SP, et al. Mechanical properties of primate vascularized vs. nonvascularized patellar tendon grafts; changes over time. J Orthop Res 1989;7:68–79.
19. Yamamoto E, Tokura S, Yamamoto N, Hayashi K. Mechanical properties of collagen fascicles from in situ frozen and stress-shielded rabbit patellar tendons. Clin Biomech (Bristol, Avon) 2000;15:284–91.
20. Ballock RT, Woo SL, Lyon RM, Hollis JM, Akeson WH. Use of patellar tendon autograft for anterior cruciate ligament reconstruction in the rabbit: a long-term histologic and biomechanical study. J Orthop Res 1989;7:474–85.
21. Cabaud HE, Feagin JA, Rodkey WG. Acute anterior cruciate ligament injury and augmented repair: experimental studies. Am J Sports Med 1980;8:395–401.
22. Chen WM, Chen TH, Huang CK, Chiang CC, Lo WH. Treatment of malignant bone tumours by extracorporeally irradiated autograft-prosthetic composite arthroplasty. J Bone Joint Surg Br 2002;84:1156–61.
23. Rasmussen TJ, Feder SM, Butler DL, Noyes FR. The effects of 4 Mrad of gamma irradiation on the initial mechanical properties of bone-patellar tendon-bone grafts. Arthroscopy 1994;10:188–97.
24. Roberts TS, Drez D Jr, McCarthy W, Paine R. Anterior cruciate ligament reconstruction using freeze-dried, ethylene oxide-sterilized, bone-patellar tendon-bone allografts: two year results in thirty-six patients. Am J Sports Med 1991;19:35–41.
25. Sterling JC, Meyers MC, Calvo RD. Allograft failure in cruciate ligament reconstruction: follow-up evaluation of eighteen patients. Am J Sports Med 1995;23:173–8.
26. Sugiura H, Sato K, Miura T, Nakanishi K, Rong Y. Tendon insertions onto allografts pretreated with heat and/or bone surface demineralization. Clin Orthop 1993;289–94.
27. Watanabe H, Ahmed AR, Shinozaki T, Yanagawa T, Terauchi M, Takagishi K. Reconstruction with autologous pasteurized whole knee joint. II. Application for osteosarcoma of the proximal tibia. J Orthop Sci 2003;8:180–6.
28. Araki N, Myoui A, Kuratsu S, Hashimoto N, Inoue T, Kudawara I, et al. Intraoperative extracorporeal autogenous irradiated bone grafts in tumor surgery. Clin Orthop 1999;196–206.
29. Diehl P, Schmitt M, Schauwecker J, Eichelberg K, Gollwitzer H, Gradinger R, et al. Effect of high hydrostatic pressure on biological properties of extracellular bone matrix proteins. Int J Mol Med 2005;16:285–9.
30. Masson P, Tonello C, Balny C. High-pressure biotechnology in medicine and pharmaceutical science. J Biomed Biotechnol 2001;1:85–8.
31. Schauwecker J, Wirthmann L, Schmitt M, Tuebel J, Magdolen U, Gradinger R, et al. Effect of extracorporeal high hydrostatic pressure on cellular outgrowth from tumor-afflicted bone. Anticancer Res 2006;26:85–9.
32. Diehl P, Naal FD, Schauwecker J, Steinhauser E, Milz S, Gollwitzer H, Mittelmeier W. Biomechanical properties of articular cartilage after high hydrostatic pressure treatment. Biomed Technik 2006;51:(in press).