J Mater Sci: Mater Med (2007) 18:303–308
DOI 10.1007/s10856-006-0693-6

Tissue engineered intervertebral disc repair in the pig using injectable polymers

P. A. Revell · E. Damien · L. Di Silvio · N. Gurav · C. Longinotti · L. Ambrosio

Received: 9 November 2006 / Accepted: 7 December 2006

Abstract The intervertebral disc (IVD) has a central nucleus pulposus (NP) able to resist compressive loads and an outer annulus fibrosus which withstands tension and gives mechanical strength. The tissue engineering of a disc substitute represents a challenge from mechanical and biological (nutrition and transport) points of view. Two hyaluronan-derived polymeric substitute materials, HYAFF®120, an ester and HYADD®3, an amide were injected into the NP of the lumbar spine of female pigs (11.1 ± 1.0 Kg) in which a nucleotomy had also been performed. Homologous bone marrow stem cells, obtained from the bone marrow three weeks before spinal surgery, were included in the HYADD® 3 material (1×10^6 cells/ml). Two lumbar discs were operated in each animal. Control discs received a nucleotomy only. The animals were killed after 6 weeks and the lumbar spines recovered for histopathological study.

Nucleotomy resulted in loss of normal IVD structure with narrowing, fibrous tissue replacement and disruption of the bony end-plates (4/4). By contrast, both HYAFF®120 (4/4) and HYADD 3®(4/4) treatment prevented this change. The injected discs had a central NP-like region which had a close similarity to the normal biconvex structure and contained viable chondrocytes forming matrix like that of normal disc.

P. A. Revell (✉) · E. Damien
Biomaterials and Tissue Engineering, UCL Eastman Dental Institute, London WC1X 8LD, UK
e-mail: parevell@btinternet.com

L. Di Silvio · N. Gurav
Biomaterials Science, King' College London, Guy's Campus, London SE1 9RT

C. Longinotti
Fidia Advanced Biopolymers, 35031 Abano Terme, Italy

L. Ambrosio
Institute of Composite and Biomedical Materials-C.N.R, 80125 Naples, Italy

1 Introduction

The intervertebral disc (IVD) consists of an internal semi-fluid proteoglycan (PG) rich mass containing chondrocytes, the nucleus pulposus (NP), and an outer annulus fibrous (AF) with concentric layers of collagen alternately angled across each other and embedded in PG also containing chondrocytes. The nucleus pulposus has a large swelling pressure and the proteoglycan matrix, comprising hyaluronan with chondroitin and keratan sulphates, is extremely hydrophilic [1, 2]. The water content of the disc varies between 65 and 85 per cent. The nucleus pulposus has turgidity so resists and distributes compressive forces, while the fibrous annulus withstands tension and provides mechanical strength and stability. The orientation of collagen in the annulus fibrosus has an important influence on the load distribution, the fibres being arranged as concentric lamellae, inclined with respect to the vertical axis of the spine in a lay-up pattern [3]. The fibres are angled from 62 degrees in the outer part of the annulus to 45 degrees near the nucleus pulposus [2]. The intervertebral disc is anchored to the vertebral bodies mainly through the peripheral part of the annulus fibrosus [4] . The periphery of the AF contains type I collagen fibres, whereas the inner AF and the NP contain type II collagen [2, 5]. The intervertebral disc is avascular, the cells obtaining their nutrition by a diffusion process from vessels in the related bone. Thus, the biomechanical and transport properties are complex and the tissue engineering of a disc substitute represents a challenge for biomaterial and medical scientists [6].

This paper reports experiments in which the reproduction of the material and mechanical as well as the biological properties of the nucleus pulposus was attempted using two injectable polymeric NP substitute materials. HYAFF® 120 and HYADD®3 are an ester and a dodecylamide respectively, each derived from hyaluronan. These materials are manufactured by Fidia Advanced Biopolymers. Both have been shown to be non-toxic in cell culture studies [7]. Both materials showed gel-like and pseudoplastic behaviours. HYAFF® 120 is a hyaluronan based photo-linked derivative obtained by linking the hyaluronic acid molecule with a compound that initiates polymerization upon exposure to the UV light. HYADD® 3 is suitable as a vehicle or scaffold to carry cells for tissue engineering using a cell loading approach. Experimental implantation procedures were performed to examine the effects of intervention with these two materials on the repair of damage to the pig IVD resulting from nucleotomy.

2 Materials and methods

All experimental animal procedures were performed under the appropriate UK Home Office project and personal licences according to the Animals (Scientific Procedures) Act 1986. There was also local ethical committee approval of the experimental surgical procedure and a formal statistical review of the experimental design (see below).

Six female Large White/Landrace cross pigs (11.1 ± 1.0 Kg) were used. General anaesthesia was provided using atropine (0.05 mg/Kg im), stresnil (0.25 mg/Kg im), ketamine (10–15 mg/Kg iv, by slow infusion), caprofen (4 mg/Kg im), clamoxyl (0.5 ml/5 Kg im) with isofluorane/O_2 and N_2O for maintenance inhalation general anaesthesia. Blood oxygen saturation, temperature, respiratory and pulse rates were monitored continuously throughout the operative procedure. A warm pad was used beneath the animal throughout the procedure. Under fully sterile conditions, the spine was approached through an incision on the flank of the pig with dissection down through the muscle layers to the lateral side of the lumbar (L1–L5) vertebral bodies and the related IVDs. A 16 gauge hollow trochar and cannula was inserted into the side of an IVD until the NP was reached and suction applied, removing 0.5–1 ml of viscous opalescent fluid, and thus causing the effective removal of NP (nucleotomy). Nucleotomy was performed at L 1–2 and L 3–4 levels. After withdrawal of the trochar the nucleotomised discs were then injected through the cannula with substitute material (0.5–1 ml) using HYAFF® 120 or HYADD® 3 containing 1×10^6 autologous stem cells/ml. Untreated nucleotomised discs acted as controls (sham treated). The wound was sutured closed after infiltration of the deep and subcutaneous tissues with local anaesthetic to decrease post-operative pain. Temgesic was administered as required post-operatively for up to a week. Carprofen was given on days 2, 3 and 4 post-operatively, and as required on review thereafter. Clamoxyl was continued postoperatively on days 3, 5, 7, 9, 11 and 13.

Autologous stem cells (SC) were obtained by culturing bone marrow cells in DMEM containing 4500 mg/L glucose, 1 μg/ml Penicillin/Streptomycin and 20% FCS. The bone marrow to provide these stem cells was obtained under general anaesthesia from the iliac crest of the same pig using a stainless steel trephine three weeks before the nucleotomy and NP injection procedure. The anaesthetic and post-operative regimes were as described above.

The design of the experiment was such that for both materials one animal received an upper disc injection while the lower disc was left untreated, another animal received a lower disc injection and the upper disc was left, and in the third animal, both upper and lower discs were injected. This gave rise to equal numbers (4) of injected discs for each material and 4 untreated discs, with an even distribution as between upper (L1–2) and lower (L3–4) disc levels. Animals were killed 6 weeks after the operative procedure. The lumbar spine was recovered intact for pathological examination and fixed in 10% formal-saline. Parallel slices of the spine were made in the dorso-ventral (sagittal) plane using an Exakt saw and these were photographed before being prepared for histological examination using decalcified paraffin wax embedded sections (7 μm) which were stained by the haematoxylin/eosin and toluidine blue methods. Each disc was examined at two different levels and deeper sections at each level. Sections were examined in a Leica DM5 light microscope with compensated (λ/4) polarization microscopy. Digital photomicrography was performed for each disc examined.

3 Results

3.1 Animal welfare

No intra-operative complications occurred with HYAFF® 120 or HYADD® 3. There were no changes in the monitored physiological measurements (oxygen saturation, temperature, respiratory and pulse rates) during nucleotomy or the injection process. All the animals made a good post-operative recovery from the procedure whichever experimental treatment they had received and were generally fully mobile within 4 hours. There were no incidents of wound infection or dehiscence, and the animals were in normal health, putting on weight and socializing within the groups in which they were kept after an initial recovery and wound healing period. By 6 weeks post-operatively, the animals had grown to 50 Kg body weight, the largest size permitted in the indoor animal facility.

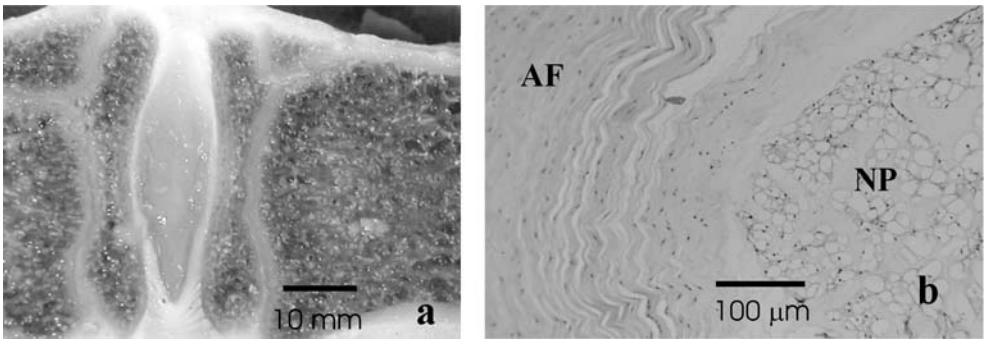

Fig. 1 Normal disc adjacent to NP substitute (HYAFF®120) injected disc after 6 weeks showing: (a) macroscopical appearance, note normal gelatinous grey central nucleus pulposus material with white annulus fibrosus and intact bony end-plate (b) histological appearance of the edge of the nucleus pulposus (NP) (right) and the annulus fibrosus (AF) (left). Note numerous chondrocytes in NP and numerous concentric layers of collagen in the fibrocartilage of AF

3.2 Normal intervertebral discs

The normal lumbar intervertebral disc is the same in appearance at all levels. After sagittal sectioning, the disc is seen as a biconvex structure between the adjacent vertebral bodies. The outer annulus fibrosus is clearly distinguishable from the softer more amorphous nucleus pulposus on naked eye examination. All the IVDs adjacent to nucleotomised and/or injected discs were examined macroscopically and histologically. All showed completely normal appearances with a central NP comprised of gelatinous appearing grey central matrix and a surrounding white fibrous annulus. On histology the former showed clusters of small chondrocytes with related matrix and this was surrounded by a more fibrocartilaginous multilayered AF. The bony end-plates of the vertebrae in relation to these normal discs were intact. There was thus no evidence that there was any effect of the operative interventions on the spine at other levels. The appearances are illustrated in Fig. 1.

3.3 Operated intervertebral discs

Macroscopic examination showed narrowing of the disc space at sites where a *nucleotomy* had been performed but no injection had been made (Fig. 2(a)). There was loss of the normal biconvex appearance and the disc had parallel straight sides where they contacted the adjacent vertebral bodies. The tissue was a homogenous white or yellowish white in appearance and no residual nucleus pulposus could be distinguished. On microscopy, the disc was replaced by vascular fibrous tissue with complete disruption of the bony end-plates and extension of the fibrous tissue from the disc into the adjacent vertebral body (Fig. 2(b)).

3.4 HYAFF®120 injected nucleotomised discs

Discs which had received a nucleotomy and been injected with HYAFF®120 retained a considerable resemblance to normal, with some retention of biconvexity (Fig 3(a)).

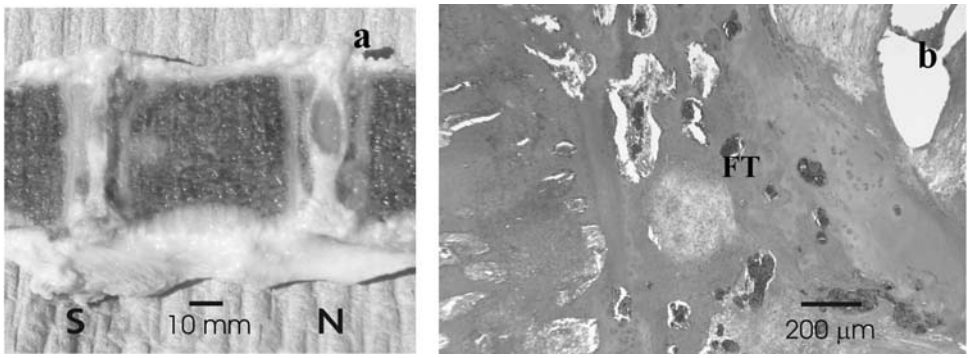

Fig. 2 (a) Nucleotomised (sham treated (S)) disc after 6 weeks; note, the narrowing of the disc space and the lack of central grey nucleus pulposus material which is present in the adjacent normal (*N*) disc (b) microscopical appearance of the disc space 6 weeks after nucleotomy. There is gross disruption of the disc with dense fibrous tissue (FT) containing numerous vessels. Little evidence of the original disc structure remains. The residual cartilage and bone of the end plates may be seen centre left and towards the top right

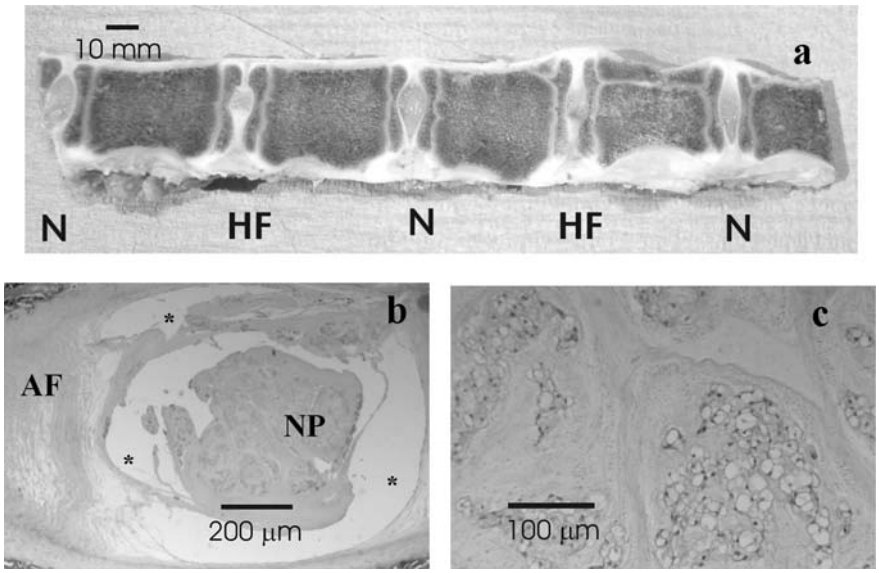

Fig. 3 (a) Appearance of HYAFF® 120 (HF) injected and adjacent normal (N) discs 6 weeks after injection treatment of nucleotomised discs. Note biconvexity of normal discs and some peripheral narrowing of treated (HF) discs, but retention of central grey coloured gelatinous material and biconvexity in the central part of the disc. (b) Low power microscopical appearance of the HYAFF® 120 treated disc shown on the left in Fig. 3(a); with some clefts in the highly cellular nucleus pulposus (NP) region and annulus fibrosus (AF). The large spaces (*) are artefacts of tissue processing. (c) High power microscopical appearance of the nucleus pulposus area shown in Fig. 3(b). Note the presence of clusters of viable chondrocytes and formation of matrix

Histological examination showed a large amount of cellular tissue with chondrocytes producing matrix present in the centre of the disc (Fig. 3(b)). There was no evidence of a toxic effect of HYAFF®120 in any of the discs. Evidence of disc regeneration was seen with retention of the normal disc space in all the discs, in contrast to the narrowed nucleotomised discs. The bony end plate was occasionally breached in some of the discs but there was no evidence of the development of vascular fibrous tissue invading the disc from the adjacent bone and the bony end plates were intact.

3.5 Cell-loaded Hyadd®3 treated disc

All four discs treated with cell-loaded HYADD®3 after nucleotomy showed some narrowing but there was a central expanded (biconvex) area in the NP region (Fig. 4). This was

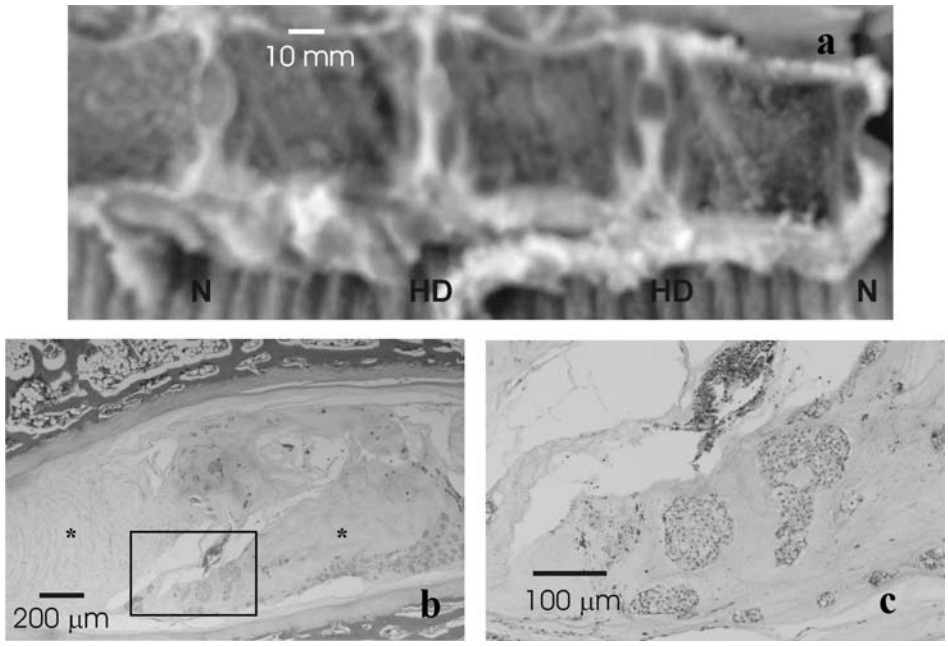

Fig. 4 (a) Macroscopical appearance of the spine 6 weeks after injection of cell-loaded HYADD®3 (HD) showing retention of some central nucleus pulposus features, particularly on the HD disc to the right. Note the normal discs (N). (b) is a low power view of the left HD disc shown in Fig. 4(a). Although the disc is somewhat disorganised, there is increased cellularity, with different patterns present. There are areas resembling normal NP. The box area is shown at higher power in Fig. 3(c). Elsewhere in this disc smaller cells are present (centre top, Fig. 4(c)). There are two areas of matrix in which cells are sparse (*) (see Fig. 4(b))

pale grey to grey-olive coloured in the different discs. The appearance was intermediate between the nucleotomy and normal appearances. By light microscopy, the cell-loaded HYADD®3 treated discs showed good retention of disc architecture with a central cellular NP zone clearly defined and biconvexity to the overall disc shape. Some of the cells in the central area showed a close resemblance to normal NP (Fig. 4, compare with Fig. 1) while in other sites chondrocytes were smaller and in linear and cribriform patterns in a looser matrix (Fig. 4). Some areas were relatively lacking in cells and showed loose matrix only. There was no evidence of damage to the adjacent vertebral bodies and the bony end plates were intact. There was no necrosis or inflammation in any of the discs.

3.6 Statistics

Prior statistical analysis showed that there was an 80% probability of showing a statistically significant result at the 95% confidence level using the clear but simple indicators of outcome and the experimental design chosen (i.e. the presence or absence of different features). Indeed, although the group sizes are small, the results are highly significant since living cells, matrix production and retention of normal NP overall shape, endplate integrity, fibrous tissue replacement of the disc, vascularisation of the disc were all scored as present or absent in all samples receiving both experimental materials and the control nucleotomised discs. The results of this statistical analysis are shown in Table 1. χ^2 values were 8 where the feature was present in all four samples in a group and absent from all four samples of the comparative group, while χ^2 values were 4.8 where the feature was present 3 out of 4 times compared with absence in all four discs in a particular group. Thus, living chondrocytes, matrix production, biconvex shape, NP/AF differentiation, and end-plate integrity were present in the treated and absent from the untreated discs, while fibrous tissue replacement and vascularisation of the disc were present in the nucleotomised discs and absent from the treated ones, whether with HYAFF® 120 or HYADD®3.

Table 1 Statistical significance of differences between treatment groups and nucleotomy controls (p values)

	Treatment group compared to control	
FEATURE	HYAFF®120	HYADD®3
Living cells	<0.01	<0.01
Matrix production	<0.05	<0.05
Biconvex shape	<0.01	<0.01
NP & AF differentiation	<0.01	<0.01
Endplate integrity	<0.05	<0.01
Fibrous tissue replacement	<0.01	<0.01
Vascularisation of disc space	<0.01	<0.01

($\chi^2 = 8$; $df = 1$; $n = 4$; $p < 0.01$).
($\chi^2 = 4.8$; $df = 1$; $n = 4$; $p < 0.05$).

4 Discussion

The experiments reported clearly show the damaging effect of nucleotomy on the pig intervertebral disc. When nucleotomy is carried out, intervertebral disc degeneration is inevitable [8]. Lack of the nucleus pulposus with its turgidity and resistance to compressive forces results in breakdown of the adjacent annulus fibrosus and bony endplates. Preserving the disc structure, particularly the nucleus pulposus, has been shown to result in better clinical results [9]. According to the present findings, healing after nucleotomy occurs by the formation of vascular fibrous tissue derived from the adjacent vertebral body and reaching the disc space through ruptures in the bony end plates. The disc however becomes thinner than the normal structure and is not able to respond to compressive and other forces.

The aim of the research reported in this paper was to develop an injectable hydrogel which was biocompatible and bioactive. The materials used needed to allow appropriate transport of nutrients to the disc chondrocytes and have the correct rheological properties. Since the NP matrix is predominantly hyaluronan, it seemed appropriate to experiment with substances related to and derived from hyaluronan. Hyaluronan itself has been shown to have a beneficial effect in a monkey nucleotomy model as assessed by radiographic imaging methods [10]. The present study is the first to report the detailed histological appearances of the repaired intervertebral disc after nucleotomy using hyaluronan related materials.

Screening procedures in which osteoblasts and other cells were cultured on HYAFF®120 and HYADD® 3 showed good viability [7]. The *in vivo* injection studies reported here show that both materials are able to support cell growth and matrix formation in the loaded intervertebral disc. In the case of HYAFF®120, the cells must have been derived from chondrocytes already present in the disc, either from the inner part of the annulus fibrosus or from residual nucleus pulposus. In this respect it is interesting to note observations by Taylor and colleagues who showed that the transitional zone between NP and AF was the main site of cell proliferation in the normal IVD [11]. It seems unlikely that the whole of the nucleus pulposus was removed by the procedure used but it is also noteworthy that whatever cells may have remained, these were not able to regenerate the nucleus pulposus after nucleotomy. This difference between experimentally injected and control (nucleotomy without treatment) discs clearly shows the need for a matrix with suitable mechanical and biological (diffusion) properties.

The presence of disc chondrocytes in an unfavourable mechanical environment almost certainly means that repair cannot take place in the absence of a substitute material.

The successful repair achieved with HYADD®3 is partly a reflection of the prior careful in vitro studies with bone marrow stem cells and this material (7). It is not known at this stage whether this success with HYADD®3 is merely a reflection of its material and mechanical properties or whether there was survival of and a contribution by the stem cells. That bone marrow derived precursor cells undergo chondrogenic differentiation in a culture system like that used for the present experiments has been shown previously [12]. Further long term studies are required to determine which of the two approaches is most likely to give the best long-term effect. However, the ability to use a material which will be effective without the addition of homologous bone marrow derived stem cells has attractions both from the point of view of simplicity and that of cost effectiveness in the clinical environment.

5 Conclusions

There was no evidence of necrosis or inflammation on injecting IVDs with hyaluronan-related materials. Evidence of regeneration was seen with disc space retention in all treated discs examined after 6 weeks. The results showed the successful repair of the nucleotomised IVD using injectable acellular (HYAFF®120) and cellloaded (HYADD®3) materials and present the prospect of successful tissue engineering for disc disease in the future.

Acknowledgments This work was funded under the EC R&D programme by FP5 Contract G5RD-CT-2000-00267. The technical support of D Moore, T McFarlane and T MacInnes is gratefully acknowledged.

References

1. T. E. HARDINGHAM and H. MUIR, *Biochim. Biophys. Acta* **279** (1972) 401.
2. D. K. OSEI, Ph D thesis, University of London 1994.
3. D. S. HICKEY and D. W. L. HUKINS, *J. Anat.* **131** (1980) 81.
4. H. INOUE, *Spine* **6** (1981) 139.
5. D. J. PROCKOP, K. I. KIVIRRIKO, L. INDERMAN and N. A. GUZMAN, *N. Engl. J. Med.* **301** (1979) 13.
6. Q. B. BAO, G. M. MCCULLEN, P. A. HIGHAM, J. H. DUMBLETON and H. A. YUAN, *Biomaterials* **17** (1996) 1157.
7. L. DI SILVIO, in "Novel Intervertebral Disc Prostheses", 3rd six monthly report. Edited by L. Ambrosio and E. Milella. EC R&D project, G5RD-CT-2000-00267, 2003. p. 66.
8. M. SATO, T. ASAZUMA, M. ISHIHARA, T. KIKUCHI, M. KIKUCHI and K. FUJIKAWA, *Spine* **28** (2003) 548.
9. J. MOCHIDA, K. NISHIMURA, T. NOMURA, E. TOH and M. CHIBA, *Spine* **21** (1996) 1556.
10. M. PFEIFFER, U. BOUDRIOT, D. PFEIFFER, N. ISHAQUE, W. GOETZ and A. WILKE, *Eur. Spine J.* **12** (2003) 76.
11. T. K. F. TAYLOR, P. GOSH, G. R. BUSHELL and J. M. SUTHERLAND, in "Scoliosis" edited by P. A. Zorab (Academic Press, London, 1977) p. 231.
12. J. U. YOO, T. S. BARTHEL, K. NISHIMURA, L. SOLCHAGA, A. I. CAPLAN, V. M. GOLDBERG and B. JOHNSTONE, *J Bone Joint Surg (Am)* **80** (1998) 1745.