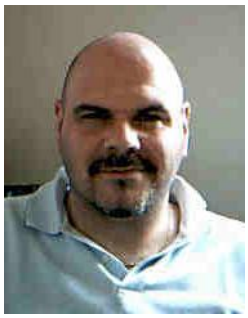
Intervertebral disc regeneration: morphological investigation of an in vitro reconstructed tissue



A. Asti[1], A.Riccardi[1], E.Benzoni[1], M. Faustini[1], E.Munari[1], P.Gaetani[2], R. Rodriguez Y Baena[2], D. Levi[2], F. Tancioni[2], E. Aimar[2], M.L.Torre[3] and D.Vigo[1]

*- massimo.faustini@unimi.it
[1] - University of Milan, Milan, Italy
[2] - IRCCS Istituto Clinico Humanitas Rozzano (MI), Italy
[3] - University of Pavia, Pavia, Italy

Introduction

Degeneration of the intervertebral disc is a widespread pathology and contributes to spondyloarthrosis, myelopathy and radiculopathy; from a histologicaly point of view, it is marked by the loss of disc height and a blurring of the gross morphologic characteristics of the annulus; the nucleus pulposus (NP) is also marked by a decline in cellularity. The current treatment of this disease consists in symptomatic pharmacological therapies or the surgical exeresis in the case of disc herniation. Recent advances in cell therapy foresee the possibility of regenerating the damaged intervertebral disc instead of its exeresis (Evans et al., 2006). Restoring cell number could be achieved by either stimulating the division and inhibiting the death of endogenous cells or by introducing new cells into the disc. In the latter case, three-dimensional culture systems offer a promising approach for obtaining *in vitro* tissues suitable for transplantation (Mizuno et al., 2004;Gruber et al, 2004). The aim of this work is to verify if a previously developed encapsulation technique for spermatozoa, granulosa and oocyte culture and preservation (Conte et al,1999, Vigo et al., 2004) could also be suitable for NP cells.

Materials and methods

Patients

Ten patients (6 males and 4 females, age 44 ± 6 years) were enrolled for the study. All the patients were admitted with diagnosis of symptomatic lumbar disc prolapse and were operated on within 3-6 months from the beginning of radicular symptoms. In all cases patients underwent a standard microdiscectomy with interlaminar approach. In 5 cases the disc prolapse was at L5-S1 level, in other 5 cases at L4-L5 level. In 4 cases the disc prolapse was completely extruded.

T*issue processing and cell culture*

Tissues excised during surgery was minced in fragments of about 1-2 mm^3 and submitted to digestion treatment with pronase for 60' at 37°C (digestion medium: DMEM-Ham's F12, 5% FBS and 1% Penicilline-Streptomicine) followed by digestion with collagenase (digestion medium described above) overnight at 37°C. After incubation, cells were filtered through a sterile 70µm filter and harvested by centrifugation (800xg for 5'); cell count and was assessed with the Trypan blue method. The pellet was divided in two aliquots for monolayer and 3D culture, respectively. Monolayer culture was performed plating 1x 10^5 cells/well (1000 µl of culture medium DMEM-Ham's F12, 10% FBS, 1% Penicillin-Streptomycin, 1% Nystatin). The capsules for 3D cultures ($1x10^5$ cell each) were prepared by using a "coat-core" method previously described (Vigo et al, 2004.); briefly, the pellet was diluted, added of a saturated solution of $BaCl_2$ and extruded through a needle into a medium viscosity 0,5% w/v sodium alginate solution. The capsules were then collected and suspended in the culture medium, one capsule for well. All media and reagents were purchased by Sigma-Aldrich (Milano-Italy)

Light and electron microscopy
After one week, the monolayer and 3D cell cultures were processed by immersion in a mixture of 2.5% glutaraldehyde (Fluka, Milano, Italy) and 0.1 mol/L Na cacodylate (Sigma-Aldrich, Milano, Italy) buffered at pH 7.4 for 90 min and post-fixed (90 min) in 0.1% osmium tetroxide (Electron Microscopy Sciences, Roma, Italy) in the same buffer at room temperature. Dehydration was performed in serial ethanol (Sigma-Aldrich, Milano, Italy) concentrations, from 50% to 100%, 15' each passage. Samples were impregnated with propylene oxide for 30' and then with solutions of propylene oxide/resin, and finally embedded in EPON 812 (Epoxy resin, Electron Microscopy Sciences, Roma, Italy). Ultrathin and semithin sections were obtained by an ultra-microtome (Ultracut E ultramicrotome, Reichert Jung, Agere Systems Analytical Lab, Orlando, FL). Semithin sections were stained with Toluidine blue and observed under light microscope. The ultrathin sections were stained with uranyl acetate and lead citrate (Fluka, Milano, Italy); ultrastructural observations and micrographs were made with a Jeol JEM 1200EX transmission electron microscope (JEOL Italia S.p.A., Milano, Italy) operating at 80kV.

Results and Discussion

Histological images show a morphologically heterogeneous cell population: both in monolayer and 3D culture (figure 1) rounded and spindle-shaped cells can be observed and cells appear embedded in an extracellular matrix which has been synthesized during the incubation. In monolayer culture, both kind of cells seem randomly distributed in clusters, with a roughly spread matrix, often incomplete, around the cells (figure 1a).

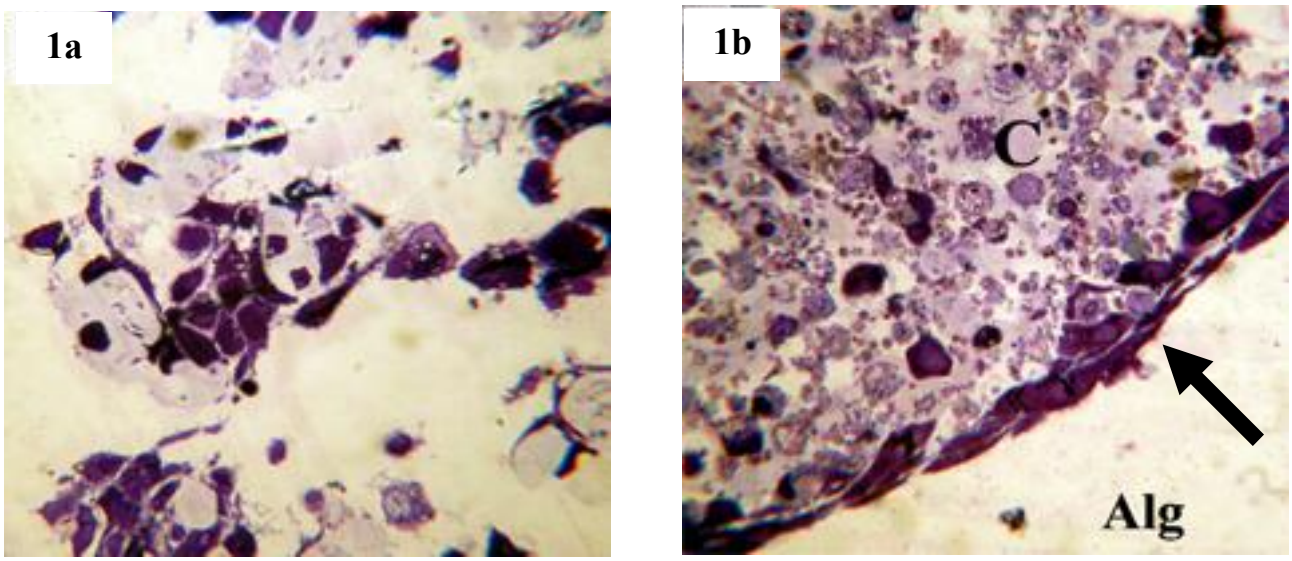


Figure 1 - Light micrograph of nucleus pulposus cells after 1 week of culture: matrix that contains sulfated glycosaminoglycans appears pink and cell nuclei are purple. (a) monolayer culture; (b) three dimensional culture in capsule. C: capsule core; Alg: barium alginate membrane; arrow: spindle-shaped cells laying on the core/membrane interface. Blue toluidine stain; original magnification: 100x.

Three-dimensional culture (figure 1b) presents a fairly different structure: the capsule appears as a cell-containing core ('C' in figure 1b) with a surrounding alginate membrane ('Alg' in figure 1b). The round-shaped cells are uniformly distributed in the core embedded in an homogeneous intercellular matrix; the spindle-shaped cells lay on the core-alginate interface (arrow in figure 1b), which acts as a basal membrane. Similar results were reported by Lee et al. (2006) on rat articular

cartilage cells and Yates et al. (2004) on bovine chondrocyte 3D culture. On the scaffold surface, cells have a flat and elongated morphology and are densely aggregated without a metachromatic extracellular matrix.
We speculate that the inhomogeneous structure of the capsule, constituted by a liquid core and a hydrogel membrane can promote spindle-shaped cell migration and organization; a similar behaviour has been previously observed for granulosa-oocyte coculture (Torre et al., 2006). Although the assessment of extracellular matrix was not quantitative, on the basis of its distribution around and between cells, and of their spatial distribution, it can be argued that barium alginate gel capsules support extracellular matrix production.

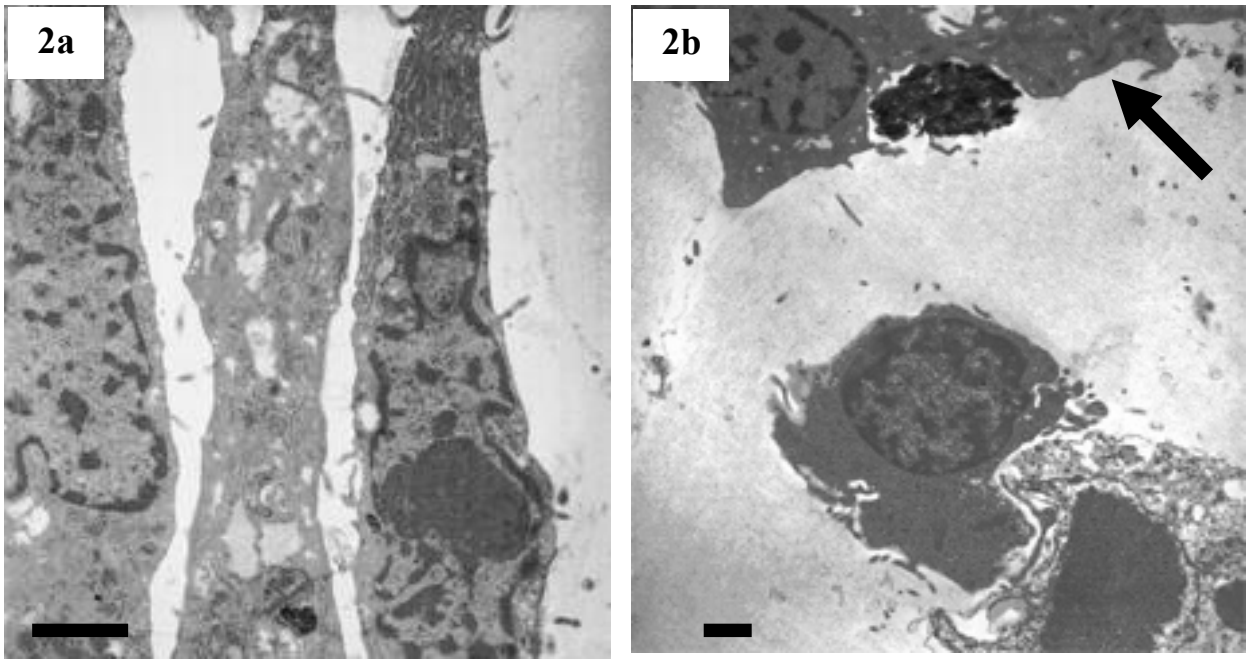


Figure 2 - TEM microphotograph of nucleus pulposus cells after 1 week of monolayer culture. 2a: fibroblast-like cells; 2b: macrophage (arrow) surrounding a particle, probably collagen. Bar: 1 μm.

The ultrastructural investigation of the monolayer cultured NP cells show a fibroblast-like morphology with elongated nucleus and rough endoplasmic reticule (figure 2a); macrophages have been observed (figure 2b): pseudopodia, associated with this cell during its active state, are used for motility as well as phagocytosis; it is believed that infiltration by various cells such as vascular endothelial cells, lymphocytes and macrophages, occurs during the course of intervertebral disc degeneration.
In 3D culture (figure 3), rounded cells as well as fibroblast-like cells can be appreciated: figure 3a shows a cell with an irregular nucleus, a well developed rough endoplasmic reticulum and Golgi apparatus; among the cells, the extracellular matrix can be appreciated. Similarly to the monolayer culture, fibroblast-like cells aggregates with a limited extracellular matrix are visible also in 3D culture (figure 3b).

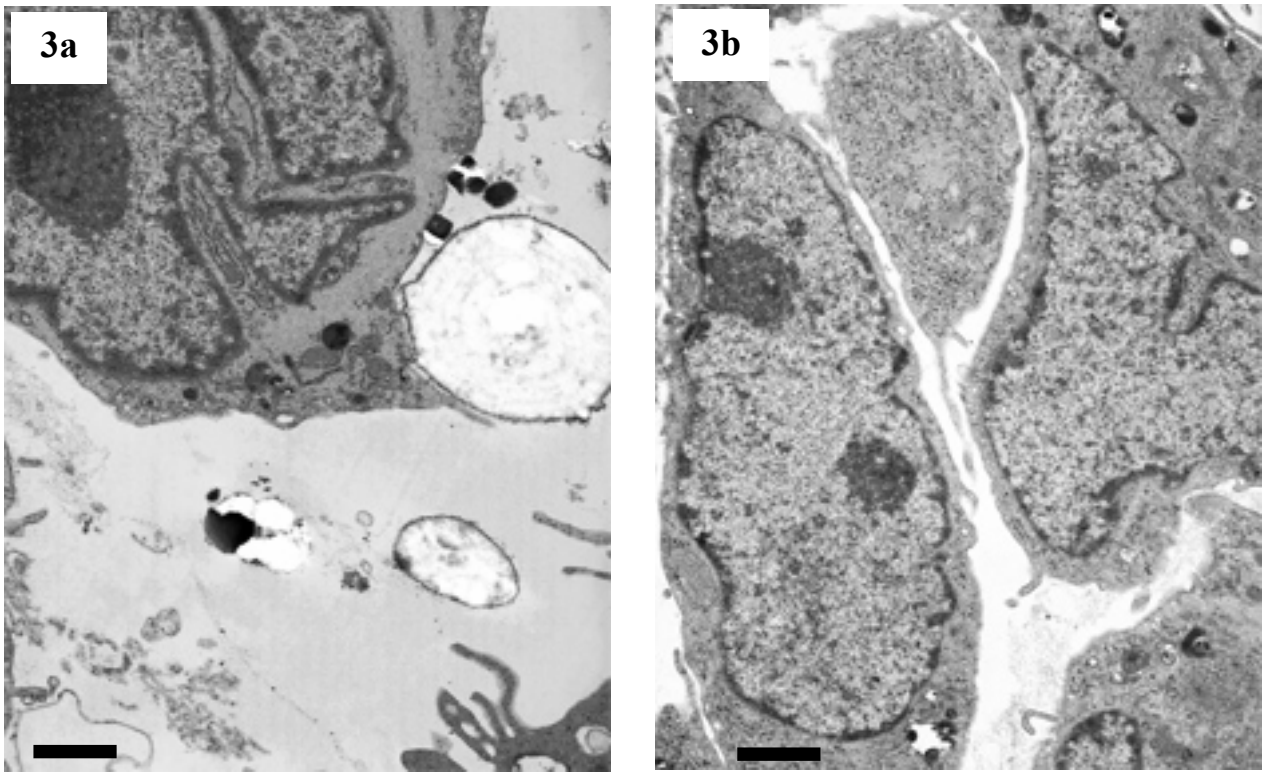


Figure 3 - TEM microphotograph of nucleus pulposus cells after 1 week of three dimensional culture in capsule. 2a: round shaped cell embedded in abundant extracellular matrix; 2b: fibroblast-like cells. Bar: 1 μm.

Conclusions

This morphological investigation confirms that the 3D culture supports extracellular matrix production by NP cells. Moreover, this "coat-core" culture system allows the 3D organization of two kinds of NP cells: matrix embedded-round-shaped cells colonize the core and the elongated cells lay on the inner alginate surface, forming a continuous layer of intermingled cells. Alginate membrane acts as a shape-forming support and provides initial mechanical stability of the cells. In conclusion, barium alginate capsules are a promising strategy to obtain a neo-tissue for regenerative cell therapy of intervertebral disc pathologies. This result is encouraging and would suggest further researches to assess cell properties (e.g. expansion, viability and phenotype), extracellular matrix composition, as well as the essential requisites for clinical applications.

References

Conte U.et al., European Patent EP0922451 (16/6/99).
Evans C. (2006). *J Bone Joint Surg* Am.88, 95.
Gruber E. et al. (2004) *Spine J.*, 4, 44.
Lee C.-T. et al. (2006) *Biomacromolecules* 7, 2200.
Mizuno H. et al. (2004) *Spine*,29,1290.
Vigo D. et al., PCT/EP 2004/012384(02/11/04).
Yates K E. et al. (2005) *Cell Tissue Bank* 6, 45.

Acknowledgements

PRIN 2004 (n. 2004074212); FIRST ex 60% Milan University; Pavia University FAR.