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Overlapping of mononuclear cells derived from bone marrow in rats' intervertebral discs: an *in vitro* study

Sobreposição de células mononucleares provenientes da medula óssea em disco intervertebral de ratos: estudo *in vitro*

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ABSTRACT

In this study, bone marrow mononuclear cells (BM-MNC) derived from rats were used in order to promote intervertebral disc regeneration. These cells were isolated after centrifugation in a Ficoll-Paque™ PLUS density gradient and then placed in plastic dishes to proliferate during a period of 14 days. The BM-MNCs were previously labeled with the fluorescent membrane marker Chloromethyl-benzamidodialkylcarbocyanine (CM-DIL), and thereafter were implanted in rats' intervertebral discs explants as an in vitro *experimental model. Daily analyses of the cells under a fluorescence microscope revealed morphological changes, which assumed a thin and elongated shape similar to cells that originally form the annulus fibroses. Histopathological analysis demonstrated the presence of mononuclear cells interspersed within collagen fibers. The presence of viable cells, in which were found morphological changes and their disposal in the same pattern of the layers that originate the annulus fibrosus, is an indicator that they engrafted and proliferated on the intervertebral disc. Therefore, morphological changes presented by these cells indicate that they presented mesenchymal stem-like cell characteristics.*

Key words: *mesenchymal stem cells, intervertebral disc,* in vitro*, rat.*

RESUMO

Neste trabalho, foram utilizadas células mononucleares provenientes da medula óssea (MO) de ratos para implantação em discos intervertebrais, a fim de estudar a sua participação em possível regeneração tecidual. Essas células foram obtidas por centrifugação, em gradiente de Ficoll-Paque™ PLUS, e cultivadas em frascos apropriados, por um período de 14 dias. Em etapa posterior, foram submetidas à marcação celular, em que foi utilizado o marcador citoplasmático CM-Dil, seguida de implantação em discos intervertebrais de ratos, em um sistema de cultivo in vitro*. Foram feitas avaliações diárias dos discos com utilização de um microscópio de fluorescência, sendo constatadas alterações morfológicas com um formato alongado semelhante a células que originalmente compõem o anel fibroso. O exame histopatológico revelou a presença de células mononucleares entremeadas em fibras de colágeno. As alterações morfológicas das células e a presença de células viáveis são indícios de que essas células se integraram e proliferaram no disco intervertebral, interagindo com o microambiente deste, assumindo inclusive características semelhantes às apresentadas por células-tronco mesenquimais.*

Palavras-chave: *células-tronco mesenquimais, discos intervertebrais,* in vitro*, rato.*

INTRODUCTION

Intervertebral disc disease is a common, frequently debilitating and painful disorder in animals. Evidences indicate that it has become the most prevalent disease in dogs. This occurrence increase may be attributed to improvements in the recognition of the disease by veterinarians, to changes in breed popularity, in which the acquisition of chondrostrofic breeds, such as Daschound, had contributed to a higher diagnosis of interevertebral diseases among these pets, and to an increase in life expectation presented by these animals due to improvements in health and overall nutritional care (BRAY & BURBIDGE, 1998a). It is the

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most common cause of paraparesis in dogs (LORENZ & KORNEGAY, 2004). In humans, degeneration of the intervertebral disc represents the major cause of low back pain and lumbar disc herniation (NORCROSS et al., 2003).

The intervertebral disc is located between each pair of vertebrae except on the atlanto-axial joint. It has three distinct anatomic regions: the annulus fibrosus, the nucleus pulposus and the cartilaginous end plates. This structure is able to resist and assure stability against deforming loads, and yet provides flexibility to the spine when these loads are within physiological limits (BRAY & BURBIDGE, 1998b; ROBERTS, 2002). The annulus fibrosus is formed by fibercartilaginous layers, which surround the nucleus pulposus, and is produced and maintained by the cellular elements located in this region. The nucleus pulposus is basically formed by water, which represents 80 to 88% of its content. Other constituents are proteoglycans which are formed by subunits of glycosaminoglycans; these are responsible for the very high negative charge imparted to the matrix of the nucleus pulposus resulting in water inflow into the discs. The presence of a high percentage of water in the nucleus pulposus allows the disc to support compressive loads (BRAY & BURBIDGE, 1998a; CREVENSTEN et al., 2004).

Disc degeneration is a progressive process and is characterized by cellular loss, degradation of the extracellular matrix, reduction of proteoglycan content, a decrease in water content and disruption of the annulus fibrosus' organized structure (BRAY & BURBIDGE, 1998a; NORCROSS, 2003; CREVENSTEN, 2004). The main therapeutic alternatives for spine diseases caused by intervertebral disc degeneration are the use of medications and surgery. However, medication does not treat the underlying cause; and surgery (disectomy, laminectomy, or fusion) does not guarantee a satisfactory result, and in most cases is associated with undesirable consequences (NORCROSS, 2003; CREVENSTEN, 2004; LORENZ & KORNEGAY, 2004).

Development of new treatments that restore disc structure and function is a challenge for bioengineering. Currently there are prosthetics that function as the disc itself but do not act on the underlying cause of the problem. Thus, a treatment that increases cellularity and restores the extracellular matrix would be a powerful alternative to treat the underlining cause of the disease (CREVENSTEN, 2004).

Stem cell research is advancing quickly, giving rise to speculation because of its potential capability to cure degenerative diseases that lead to extensive cell loss, such as the degenerative disc disease (CREVENSTEN, 2004). These cells differ from others because of three major properties: they reside in an undifferentiated state, are capable of multiplying for long periods of time through asymmetric division and are capable of differentiating in specialized cells of a particular tissue. Asymmetric division consists on the capability of the stem cell to divide and originate an identical undifferentiated cell, repairing the pool of stem cells, or alternatively, differentiating in a specialized cell (ZAGO, 2006).

Mesenchymal stem cells are multipotent cells capable of differentiating into osteoblasts, condrocytes, adipocytes, tenocytes and myoblasts among others (JOHNSTONE et al., 1998; PITTENGER et al., 1999; BARRY, et al, 2001; BARRY & MURPHY, 2004; CHO et al., 2005; COVAS, 2006; NARDI & MEIRELLES, 2006; MCTAGGART & ATKINSON, 2007). It is known that small populations of mesenchymal stem cells, corresponding to 0.001 to 0.01% of all nucleated cells, reside in the bone marrow. These cells are an important component of the mononuclear fraction present in the bone marrow (PITTENGER et al., 1999; COVAS, 2006).

The main goal of the present study was to evaluate the influence of mononuclear cells derived from rat's bone marrow, after previous culture and expansion, when implanted on rat intervertebral discs in vitro. The use of these cells is justified by their easy extraction; easy culture and the fact that they can be induced to differentiate into multiple cell lines, including condrogenic lines, when in specific media (JOHNSTONE et al., 1998; PITTENGER et al., 1999; BARRY, et al., 2001; BARRY; MURPHY, 2004; CHO et al., 2005; FORTIER, 2005; COVAS, 2006; NARDI; MEIRELLES, 2006).

MATERIAL AND METHODS

Isolation and cell culture

A pool of mononuclear cells containing mesenchymal stem cells from 3 to 4 month old Sprague-Dawley rats was isolated and cultured in "α-minimum essential media" (α-mem media)[a]. The animals used in the present study were donated and had been previously euthanized by the Department of Cellular and Molecular Cardiology from the University of California at Davis. The project was approved by the Animal Care and Use Committee of the University of California, Davis. For euthanasia, the animals were anesthetized with ketamin[b]/xilazin[c] 10mg kg^{-1} following exsanguination. Before anesthesia, a dose of heparin was applied to prevent formation of post mortem clots.

After euthanasia, the femurs where immediately dissected and the soft tissue was totally removed to guarantee an accurate extraction of the bone marrow.

Bone marrow extraction took place in a sterile hood. With a small, sterile orthopedic saw the proximal and distal extremities of the bone were removed. Curved haemostatic forceps were used to position the femur vertically inside a 5ml spinning tube. The bone marrow was removed through repeated flushing, by injecting Hank's balanced salt solution (HBSS)[d] with a 3ml syringe and 22G needle. After removing all bone marrow, it was centrifuged for 5 minutes (min) at 160xg and the supernatant was removed and the precipitated fraction resuspended in 1ml of HBSS. To this solution 0.5ml of Ficoll-Paque™ at 1.077g ml^{-1} of density[e] was added followed by centrifugation at 400xg for 15min. The result of this step was a three layered solution where the middle layer contained the mononuclear cells. An automatic pipette was used to extract the middle layer, the collected cells were submitted to cellular counting through a hemocytometer and transferred to a $25cm^2$ flask containing an adequate amount of enriched "α-minimum essential media" (α-mem media). For the culture medium preparation, fetal bovine serum, glutamine and penicillin/streptomycin were added to the α-mem media, creating a solution containing fetal bovine serum at 20%, glutamine at 1% and penicillin/streptomycin at 1%.

Finally, the flask containing the cells in culture media was transferred to a CO_2 incubator at 37°C. The media was replaced every 3 to 4 days and the cells were transferred to other flasks when the culture reached 80 to 90% of confluence. During this period the cells were evaluated and daily registered by photomicrographs.

Intervertebral disc extraction

The intervertebral discs were dissected and extracted from the tails of 3 adult rats. The animals were donated, previously euthanized, under the same conditions as described before. Immediately after the euthanasia the animals were sent to the laboratory for intervertebral disk extraction. For this procedure, the animal was placed on sternal recumbence, where its paws were tractioned and fixed with pins on a styrofoam bed.

The tail was surgically prepared using alcohol 70% followed by antiseptic povidine. In order to extract the intervertebral disc, a skin incision was made on the dorsal region of the tail and extended through its entire length. The skin was entirely removed by continuous manual traction, exposing the tail ligaments and muscle tissues. Ligaments and soft tissue were removed by blunt dissection with the help of scissors and forceps resulting in exposure of the intervertebral discs. A number 15 blade was used for delicate disc extraction; in order to do so, an incision, extending dorsal-ventrally at the endplates of each vertebra, was made. This procedure allowed loosening of the joint cartilaginous tissue from the bone. Once these discs were unattached from the tail, they were immersed in HBSS, in order to prevent dehydration.

The next step took place in the sterile hood and consisted in placing the explants in culture media. To minimize contamination they were previously treated with antibiotics (penicillin and streptomycin) for 5min. After cleansing with HBSS, the discs were transferred to a 24 well plate containing enriched a-mem media. The plate was transferred to a CO_2 incubator at 37°C where it was kept for 24 hours.

Cell labeling and superposition on intervertebral disk

Cells added to the intervertebral discs were previously labeled with the membrane probe chloromethyl-benzamidodialkylcarbocyanine (CM-DIL)[f] for further identification. For labeling, these cells underwent a process that forced them to detach from the plastic flask. This was accomplished by adding a thin layer of tripsin and EDTA directly on the cells with the use of an automatic pipette. To improve the efficiency, the flask was maintained in a CO_2 incubator at 37°C during approximately 5min. Once the cells were detached, the media containing fetal bovine serum was added, neutralizing the tripsin. The cells used in this stage had been cultivated and transferred to other flasks when necessary for at least 14 days.

After cell counting and dilution at a pre-determined concentration, the solution containing the cells was transferred to a spinning tube and centrifuged at 160xg for 5min. The remainders of the exceeding cells were placed in culture for further expansion. The precipitated fraction was resuspended in 1 ml of HBSS; the CM-DIL probe was added to this solution and left at 37°C for 10min, allowing the molecular marker to penetrate the cells. Next, the solution was centrifuged at 160xg for 5min, the supernatant was discarded and the precipitated fraction was resuspended in the culture media. The amount of media added depended on the number of cells previously present and the number of cells to be reached. For this study, the cellular concentrations used on the intervertebral disks were $1x10^3$, $1x10^4$ and $1x10^5$.

The cells were added directly on the intervertebral discs by using a 1ml syringe and 29G needle. For this step, the intervertebral discs were removed from the culture media and placed in plastic

tubes slightly larger than the discs. The tubes had two openings that allowed free access to the explants and acted as physical barriers maintaining the cells (which were diluted in media) on the discs and preventing them from having contact with the plastic plate. Nine explants were prepared and divided in 3 groups, each one receiving a different cellular concentration corresponding to $1x10^3$, $1x10^4$ and $1x10^5$. These discs were previously treated with 4'6-Diamidino-2-phenyindole (DAPI)[g], a specific marker for deoxyribonucleic acid (DNA), which allowed the identification of cell nuclei under fluorescence microscopy (ALBERTS et al, 2002). To guarantee the cell adhersion to the disc, the cells were allowed to sit for 30min before adding the culture media (enriched a-mem). This culture system was placed in a CO_2 incubator at 37° and data were harvested through daily evaluations of the explants using an inverted fluorescent microscope during 14 days.

Histopathology evaluation

The discs explants were fixed in buffered formalin, embebbed in paraffin and cut into 4µm sections with a microtome. One section was stained with hematoxylin and eosin (H&E) and the adjacent section was left unstained and mounted in Prolong Gold[h] media, supplemented with DAPI to stain nuclei. The histological sections were analyzed microscopically and photomicrographed.

RESULTS AND DISCUSSION

The formation of a three layer mixture upon centrifugation showed that the cell isolation method, based on the procedure originally described by BOYUM (1968), allowed an adequate isolation of the mononuclear cells present in the bone marrow. The middle layer, formed by mononuclear cells, was easily identified by a white halo formed between the top (platelets and proteins) and the bottom layer (erythrocytes and granulocytes). In the bone marrow, mononuclear cells are basically formed by hematopoietic stem-cells, blasts, monocytes and lymphocytes (FONTES et al., 2006). A population of rare cells, denominated mesenchymal stem cells (MSC), has been recently discovered to be mononuclear cells (JOHNSTONE et al., 1998; PITTENGER et al.; BARRY & MURPHY, 2004; COVAS, 2006; NARDI & MEIRELLES, 2006).

Isolation and cultivation of the mononuclear cells in plastic flasks for at least 14 days formed a constant flat type culture, suggesting them to be MSCs. This culture was obtained through several substitutions of the media and removal of non-adherent cells. Various studies show that MSCs, in detriment to other cell types, have great capability to adhere to plastic flasks resulting in a morphologically constant culture after 14 days (NARDI &MEIRELLES, 2006; MCTAGGART & ATKINSON, 2007).

Intervertebral disc extraction showed to be a relatively easy procedure. The use of a number 15 blade proved to be extremely important since it provided a more delicate cut, minimizing possible damage to the explants.

Inverted fluorescent microscopy of the intervertebral disks revealed cells with a spindle shaped morphology that emitted red fluorescence corresponding to the CM-DIL cytosol stainer (Figure 1A). These cells presented a thin and elongated format that resembled cells which originally form the annulus fibrosus. The same section showed presence of blue nuclei, previously treated with DAPI, which indicates viability of the intervertebral cells (ALBERTS, 2002). In addition, analyses through the inverted microscope, permitted a thorough observation of the parallel layers that form the annulus fibrosus which were characterized by variations of a light and dark green contrast (Figure 2C), similar to data provided by ROBERTS (2002). When these pictures were put together by electronic composition, a transposition pattern was evidenced, revealing the presence of injected viable pre-cultivated mononuclear cells in accordance with the disposition of the layers that form the annulus fibrosus (Figure 1D). The morphological change of the added cells and the fact that they incorporated into the annulus fibrosus suggest that these cells have interacted with the intervertebral disc's microenvironment which is a determinate factor for cell existence and function (FUCHS et al, 2004).

Cellular proliferation on disks that were treated with $1x10^3$ and $1x10^4$ cells was insignificant; and in the disks that received concentrations of $1x10^5$ cells, cellular proliferation was significant. This may be justified by the low cell number present on the previous disks, which highlights the importance of having adequate cell density in further experiments and clinical trials (GENGOZIAN, 2000). In fact, a study conducted by KERKIS et al. (2008) enhances the importance of adequate cellular concentration to achieve satisfactory clinical trial results.

Histopathology H&E stain exams of the annulus fibrosus sections revealed presence of viable cells intermingled in a rich collagenous environment (Figure 2A). An adjacent unstained section was examined and photographed under visible light. The arrows indicate presence of fluorescent cells (Figure

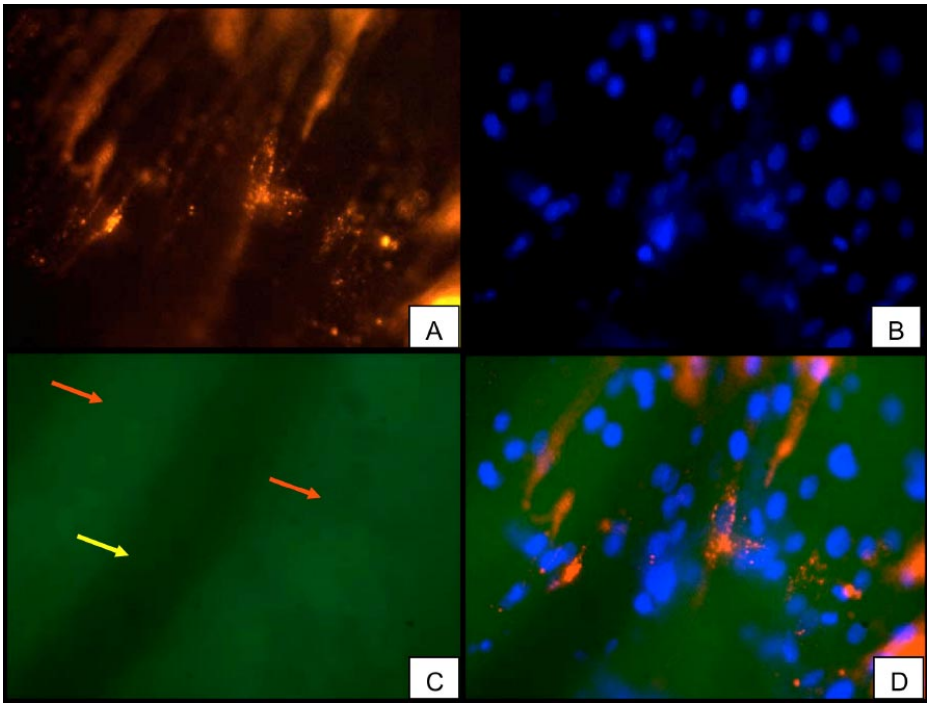

Figure 1 - Fluorescence microscopy of intevertebral disk after superposition of pre-cultivated mononuclear cells at 9 days, 600X. A) Pre-cultivated mononuclear cells implanted on intervertebral disc at 9 days, demonstrating an orange-red staining pattern (CM-DIL). B) Presence of cells stained with DAPI (cellular marker that stains the nuclei blue) C) Demonstration of the layers on the annulus fibrosus presenting a light (red arrow) and dark (yellow arrow) green contrast. D) Electronic composition of figures A, B and C revealing the presence of injected viable pre cultivated mononuclear cells following the disposition of the layers that form the annulus fibrosus.

2B), which were likely to be the implanted pre-cultivated mononuclear cells stained with the red dye (CM-DIL), since under fluorescent illumination the cytosol showed an orange-red punctuate pattern (Figure 2C). A nuclear marker (DAPI) was also included in the mounting media and stained the nuclei of all cells (Figure 2D). An electronic composition of figures 2B, 2C and 2D illustrates the relationship of the pre-cultivated mononuclear cells with the collagen matrix of the annulus fibrosus (Figure 2E). This data suggests that the cells, which were added to the disks, stopped dividing and differentiated in cells capable of integrating the annulus fibrous' microenvironment. Furthermore, morphological changes indicated that cell differentiation may have occurred, in fact these cells, which presented a flat form when on the plastic flasks, assumed a fusiform shape (similar to cells from the chondrogenic lineages, such as fibroblasts) when implanted on the explants. The evidence of the ability of these cells to integrate in the extracellular matrix also contributes to support this hypothesis. According to ALBERTS (2002), the extracellular matrix's physical and chemical properties influence the cells' differential state in connective tissues.

A tridimensional culture system was used, in which cells were stimulated to grow on intervertebral discs extracted from rats. Although it's not an ideal model, these explants were maintained in culture media (enriched á-mem) to supply nutrients for the cells. It is well-known that the nucleus pulposus contains negative charges, which generate an osmotic gradient, attracting water to the spaces between glycosaminoglycan molecules (BRAY & BURBIDGE, 1998a). This caused extrusion with significant loss of nucleus pulposus in the culture media, and this result however does not invalidate the present study since important observations in the perspective of the fibrous annulus constituent were made.

CONCLUSION

The data showed by this study strongly favor the notion that cells from bone marrow, most likely the mononuclear fraction, differentiated in condrogenic

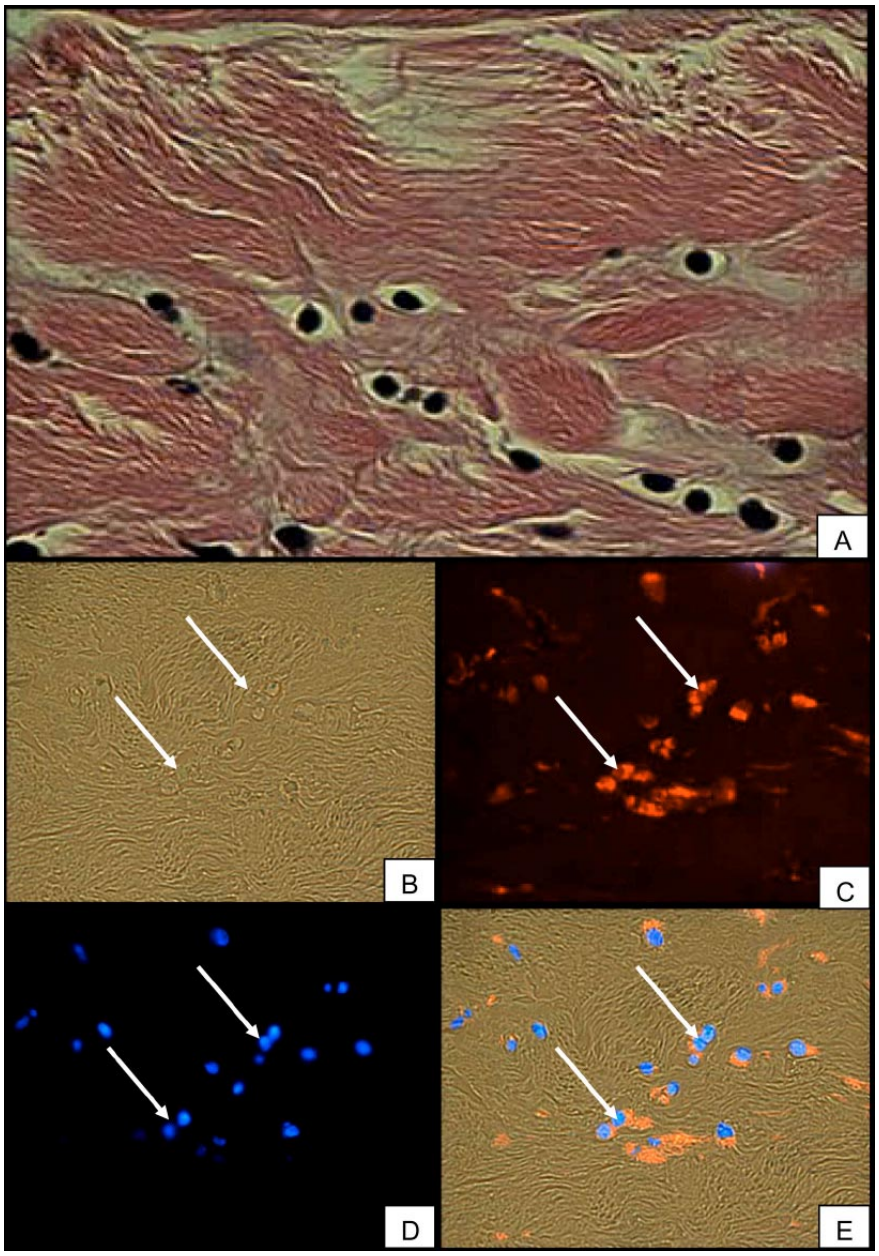

Figure 2 - Histopathology exam on annulus fibrosus section, 600X. A) H&E stain on a section of the annulus fibrosus, 600X. Presence of cells intermingled in the collagen matrix. B) Microscopy analyses in a similar field on an adjacent unstained section. Under visible light the injected cells are slightly visible (arrows). C) Fluorescence microscopy indicates an orange-red punctuate staining pattern (CM-DIL) revealing presence of injected cells. D) Fluorescence microscopy indicates presence of blue nuclei (DAPI) shown by the arrows. E) Electronic composite of figures B, C and D illustrating the relationship of the injected pre-cultivated mononuclear cells with the collagen matrix of the annulus fibrosus.

lineages when added to intervertebral disks. This was evidenced by morphological changes presented by such cells, demonstrating the presence of mesenchymal stem-like cells. Furthermore, the cellular morphological changes and the evidence of cellular viability suggest that these cells integrated and interacted with the microenvironment provided by the intervertebral disk, more specifically the annulus fibrosus.

Future studies with the use of specific markers for chondrogenic lineages are necessary not

only to show chondrogenic lineages but mainly to specify cell types, such as, chondroblast, fibroblast and osteoblast; as well as to demonstrate collagen type II expression of these cells. In addition, even if type II collagen expression is proven, collagen organization must be demonstrated, as a pre-requisite for the collagen to strengthen the intervertebral disk.

Finally, *in vivo* studies are necessary to evaluate which factors determine cellular differentiation since mechanical forces acting on the intervertebral disks may influence the kind of tissue formed by mesenchymal cells (BRAY & BURBIDGE, 1998a). Researches on *in vivo* models are needed to provide concrete data on the action of these cells and their safety when it comes to cellular therapy in clinical trials.

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SOURCES

a - Alpha Minimum Essential Media – Mediatech/Cellgro
b - Quetamina - Vetaset® - Fort Dodge
c - Xilazina - Roumpum® - Bayer
d - Hank's balanced salt solution– Mediatech/Cellgro
e - Ficoll-Paque PLUS – Amersham Biosciences
f - CM-DIL – Invitrogen/Molecular Probes
g - DAPI – Invitrogen/Molecular Probes
h - Prolong Gold - Invitrogen/Molecular Probes

REFERENCES

ALBERTS, B. **Molecular biology of the cell.** New York: Garland Science, 2002. 1463p.

BARRY F. et al. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. **Experimental Cell Research**, New York, v.268, n.2, p.189-200, 2001.

BARRY, F.P.; MURPHY, J.M. Mesenchymal stem cells: clinical applications and biological characterization. **International Journal of Biochemistry & Cell Biology,** Exeter, v.36, n.1, p.568-584, 2004.

BOYUM, A. Isolation of mononuclear cells and granulocytes from human blood. **Scandinavian Journal of Clinical and Laboratory Investigation**, Oslo, v.21, Suppl.97, p.77-89, 1968.

BRAY, J.P; BURBIDGE, H.M. The canine intervertebral disk. Part one: structure and function. **Journal of the American Animal Hospital Association,** Lakewood, v.34, p.55-63, 1998a.

_____; _____. The canine intervertebral disk. Part two: degenerative changes-nonchondrodystrophoid versus chondrodystrophoid disks. **Journal of the American Animal Hospital Association,** Lakewood, v.34, p.135-144, 1998b.

CHO, H.H. et al. Induction of osteogênic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. **Journal of Cellular Biochemistry**, New York, v.96, n.3, p.533-542, Oct. 2005.

COVAS D.T. Células-tronco mesenquimais. In: ZAGO, M.A. (Ed.); _____. **Células-tronco a nova fronteira da medicina.** São Paulo: Atheneu, 2006. Cap.3, p.35-48.

CREVENSTEN, G. et al. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. **Annals of Biomedical Engineering,** v.32, n.3, p.430-434, 2004.

FONTES, A.M. et al. Células-tronco e seus métodos de estudo. In: ZAGO, M.A. ; COVAS, D.T. (Ed.). **Células-tronco a nova fronteira da medicina.** São Paulo: Atheneu, 2006. Cap.7, p.93-106.

FORTIER, L.A. Stem cells: classifications, controversies, and clinical applications. **Veterinary Surgery**, Philadelphia, v.34, n.5, p.415-423, 2005.

FUCHS, E. et al. Socializing with the neighbors: stem cells and their niche. **Cell**, Cambridge, v.116, n.6, p.769-778, 2004.

GENGOZIAN, N. Identification and isolation of hemopoietic progenitors. In: FELDMAN, B.F. et al. **Schalm's veterinary hematology**. Philadelphia: Williams & Wilkins, 2000. Cap.16, p.91-96.

KERKIS, I. et al. Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic? **Journal of Translational Medicine**, v.6,p.35, 2008. Available from: http://www.translational-medicine.com/content/6/1/35. Accessed: 24 march 2008. doi:10.1186/1479-5876-6-35.

JOHNSTONE, B. et al. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. **Experimental Cell Research**, New York, v.238, n.1, p.265-272, 1998.

LORENZ, M.D.; KORNEGAY, J.N. **Handbook of veterinary neurology**. Philadelphia: Saunders, 2004. 469p.

MCTAGGART, S.J.; ATKINSON, K. Mesenchymal stem cells: immunobiology and therapeutic potential in kidney disease. **Nephrology**, Carlton, v.12, n.1, p.44-52, 2007.

NARDI, N.B.; MEIRELLES, L.S. Mesenchymal stem cells: isolation, in vitro expansion and characterization. **Handbook of Experimental Pharmacology**, New York, v.174, p.249-282, 2006.

NORCROSS, J.P. et al. An in vivo model of degenerative disc disease. **Journal of Orthopedic Research,** New York, v.21, p.183-188, 2003.

PITTENGER, M.F. et al. Multilineage potential of adult human mesenchymal stem cells. **Science,** New York, v.284, n.5411, p.143-147, 1999.

ROBERTS, S. Disc morphology in health and disease. **Biochemical Society Transactions**, Portland, v.30, n.6, p.864-869, 2002.

ZAGO, M.A. Células-tronco: origens e propriedades. In:_____;COVAS, D.T. (Eds). **Células-tronco a nova fronteira da medicina.** São Paulo: Atheneu, 2006. Cap.1, p.3-20.