CANINE MESENCHYMAL STEM CELLS ISOLATION FROM BONE MARROW ASPIRATES

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Abstract: Bone marrow mesenchymal stem cells are an integral part of the hemopoetic microenvironment. Due to the rarity of these cells in the bone marrow and the lack of definitive markers that specifically isolate MSCs these cells are commonly isolated by adherence to plastic and consecutive passage in tissue culture.

The aim of the present paper was to evaluate three bone marrow harvesting sites: the proximal humerus, proximal femur and the wing of the ilium and two isolation and cultivation media containing α-MEM and DMEM for mesenchymal cell culture in dogs. 3 ml of bone marrow were harvested from each puncture site by aspiration in flushing medium containing syringes for a total of 9 ml/dog. Two batches were established, batch number I using α-MEM cell culture medium and batch number II with DMEM culture medium. The most efficient bone marrow puncture and harvesting sites were the humeral one, followed by the femoral one. Comparing the two culture media used we have determined that α-MEM medium had a higher efficiency rate than DMEM upon cell plastic adherence, viability and proliferation capacities, detecting distinct mesenchymal cell colonies at 6 days of culture with homogenous fibroblast-like morphology and reaching 70% confluence at 7 days (passage 1). Cell viability after thawing was 70% for the first batch and 65% for the second one.

INTRODUCTION

The bone marrow compartment contains a population of stem cells with extensive capacities for self-renewal and proliferation called mesenchymal stem cells (MSCs) or stromal progenitor cells (Kern Susanne et all 2006). Under appropriate culture conditions these cells are able to differentiate into several types of mesenchymal cells, including osteocytes, chondrocytes and adipocytes, but can also differentiate into non-mesenchymal cells such as neural cells (Livingston Treena et all 2003, Hiroaki K. et all 2008).

Mesenchymal stem cells of the bone marrow are an integral part of the hemopoetic microenvironment, which supports blood cell development (Nan Wang. et all 2008). Without preceding selection or cellular cloning, these cells consist mainly of fibroblast-like cells. Due to their rarity and the lack of definitive markers, these cells are commonly isolated by adherence to plastic and consecutive passage in tissue culture (Le Blanc K et all 2007, Nan Wang et all 2008). MSCs may expand several fold in vitro, for instance after aspiration of bone marrow, being used for in vitro research, or clinical applications.

Many features of dogs make them suitable animal models to study new therapeutic approaches for human diseases: a large body mass, a long life span and spontaneous diseases (Hiroaki K et all 2006). Historically, the dog has been a valuable model for bone marrow transplantation studies, with many of the advantages being directly transferable to human clinical bone marrow transplantation protocols. In addition, the dogs are also the source of many well-characterized homologues of human genetic diseases, making them an ideal large animal model in which to evaluate gene therapy protocols (Mahesh H. et all 2006)
MATERIAL AND METHODS

Researches were done during March-June 2008 on 2 clinically healthy half-blood dogs of 6 and 8 months old of 8 kg respectively 15 kg belonging to Cluj-Napoca Veterinary Faculty Clinical Hospital.

Bone marrow was harvested after general anesthesia with acepromazine (Aceprom) 0,75 mg/kc and ketamine (Vetased) 10 mg/kc. The first dog was anesthetized with 6 mg of Aceprom and 80 mg of Vetased. The second dog was anesthetized with 11,25 mg of Aceprom and 150 mg of Vetased. Both dogs were locally anesthetized with alfacaine and adrenaline in separate points using 2 ml on each point. The local anesthesia was performed placing the anaesthetic agent into the skin and muscles.

The skin surface was clipped of hair and aseptically prepared as for surgery. Blood samples were collected for metabolic parameters testing: hematocrit, hemoglobin, eritrocites, urea, creatinine, ALAT, GGT, lipase, amylase, proteins, albumins and γ globulins.

We have tested the most accessible sites for bone marrow aspiration in the dog: the proximal humerus, proximal femur and the wing of the ilium, approached either from the dorsal crest or lateral face (figure 1).

Figure 1. The sites tested for bone marrow aspiration

Single use 16 and 18 gauges harvesting needles with inner stylets, essentials to penetrate the cortex of the bone, were used. Each time, a small 1 cm skin incision was made with a scalpel blade in order to penetrate easier into the bone. At the femoral election sites muscle dilacerations were performed for the same purpose.

To obtain marrow from the proximal humerus, the animals were restrained in lateral recumbency, the elbow was rotated inward in such way that the shoulder joint was turned outward. Bone marrow was obtained by running the fingers down the spine of the scapula and inserting the needle at the distal end of this bony prominence. The needle was inserted at an angle of approximately 45 degrees from a line parallel to the long axis of the humerus.

For bone marrow collection from the wing of the ilium the animals were restrained in lateral recumbency. The dorsal crests of the wing of the ilium were palpated and the punctures were made approximately 1 cm lower. The needles were directed parallel to the long axis of the wing of the ilium.

Bone marrow was obtained from the proximal femoral shaft by inserting the needle in the trochanteric fossa between the lesser and greater trochanter. The needles were kept parallel to the thumb. If the parallel line wouldn’t be maintained, the needles could exit the cranial or caudal cortex of the bone. Once the needles were in contact with the surface of the bone they were rotated with clockwise motions. The punctures were made under radiological control.

10 ml syringes were used to aspirate bone marrow. On each syringe 3 ml of flushing medium was added prior to bone aspiration. The flushing medium was composed of: DMEM
(1000) low glucose (1g/l), 10% FCS, 1% glutamine, 1% penicillin-streptomycin, 1% NEA, heparin 5U/ml.

The syringe plunger was pulled back on to 5-7 ml creating enough negative pressure to break marrow particles loose from the endosteal surface and mixing the aspirated bone marrow with the flushing medium.

Each of the 6 bone marrow samples was separated in two. Half of the samples were centrifuged for 7 minutes, 1500 rpm at 4°C using the cellular deposit. The other half were dispersed by repeated suctions and releases.

Two mesenchymal cells isolation and culture methods were used and the cells were distributed in two batches. The first batch was flushed with 5 ml Hank’s solution, then the cells were resuspended in 0.84% ammonium chloride for 5 minutes, centrifuged for 7 minutes at 1500 rpm, washed and cultured in a medium consisting of α-MEM with 15% FCS, 1% glu, 1% NEA and 1% penicillin-streptomycin.

Cells were plated in 25 cm² culture flasks and incubated at 37°C with 5% humidified CO₂. After 48 hours non-adherent cells were discarded and adherent cells were thoroughly washed with PBS. Fresh complete culture medium was added and replaced every 3-4 days. The first passage was made at 7 days when the cells reached 70% confluence. The second passage was performed after another 5 days.

The second batch was cultured in a medium consisting of DMEM (1000) low glucose with 10% FCS, 1% NEA, 1% glutamine, 1% penicillin-streptomycin. Cells were plated in 25 cm² culture flasks and incubated at 37°C with 5% humidified CO₂. After 24 and 48 hours non-adherent cells were discarded and adherent cells were washed with PBS. Fresh complete culture medium was added and replaced every 3-4 days. The first passage was made at 9 days when the cells reached 65% confluence. The second passage was performed after another 7 days.

The passages were performed using 0.25% trypsin and 1 mM EDTA at 37°C for 5 minutes. The trypsin solution was neutralised with media containing 10% FCS. Cellular suspension was recentrifugated at 1500 rpm, and the cells were collected from the sediment and plated in 25 cm² flasks.

Stem cells were frozen using the cryopreservation medium: DMEM complete medium, DMSO, FCS and Mr Frosty cooler (1 grade/minute) for gradual cooling. After 24 hours the cells were placed in a liquid nitrogen tank at –196°C.

After thawing cell viability was tested using Trypan-Blue stain and measured using Burker-Turk cell-counting plate, dead cells being stained in blue and viable cells being uncolored.

RESULTS AND DISCUSSIONS

The humerus punctures were easily performed. Caution was required to avoid needle insertion at an angle perpendicular to the humerus and the needle entry into the bicipital bursa that communicates with the scapulohumeral joint on the medial side of the limb.

The proximal femoral shafts were relatively accessible for aspiration. Muscle dilacerations were performed in order to penetrate easier into the bone. The needles had sufficient length to reach this site (a problem for larger dogs). Caution was required because the sciatic nerve is caudal to the femur and can be injured.

At the lateral wing of the ilium the marrow cavities were shallow. The needles couldn’t be seated very deeply because of the risk of passing through the marrow cavity and into or through the cortical bone on the opposite side of the wing.
Bone marrow harvesting was certified by the radiological control proving that the needles were inserted in the medullar canal (Figure 2).

Approximately 3 ml of bone marrow were harvested from each puncture site by aspiration in flushing medium containing syringes (3 ml) for a total of 9 ml/dog (Figure 3), aspiration of larger volumes determining dilutions of the bone marrow samples with peripheral blood.

If initially the cells were constituted in a suspension, shape changes in the first batch were observed after 2 days and in the second batch after 3 days. Isolated fibroblast-like cells or groups of spindle like cells were spotted at 3 days for the first batch and 5 days for the second batch (Figure 4). The mesenchymal cells colonies continued to grow, becoming distinct at 6 days of culture for the first batch and at 8 days for the second one. At 7 days the cells from first batch reached 70% confluence. The second batch achieved 65% confluence rate at 9 days (Table 1).

### Table 1.

<table>
<thead>
<tr>
<th>Detected changes</th>
<th>I BATCH</th>
<th>II BATCH</th>
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<tbody>
<tr>
<td>Shape changes</td>
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<tr>
<td>Fibroblast-like cells</td>
<td>3 d</td>
<td>5 d</td>
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<td>Distinct cell colonies</td>
<td>6 d</td>
<td>8 d</td>
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<td>1st passage</td>
<td>7 d</td>
<td>9 d</td>
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<tr>
<td>2nd passage</td>
<td>7 + 5 d</td>
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The mesenchymal cells had homogenous fibroblast-like morphology being accompanied by little round mononuclear cells (monocytes). After trypsinisation and passaging more homogenous cultures were observed (Figure 5).

Cell viability after thawing tested with Trypan-Blue stain was 70% for the first batch and 65% for the second batch.
CONCLUSIONS

- From the point of view of the approach possibilities the most efficient bone marrow puncture and harvesting sites were the humerals, followed by the femurals.
- The ilium wing puncture technique is not recommended for use in very small dogs due to the tendency to penetrate both cortices.
- For the first batch, the use of ammonium chloride permitted formation of quite homogenous cell cultures, without erythrocytes.
- The α-MEM medium favored an early plastic attachment of the bone marrow mesenchymal stem cells with the presence of a small number of cells in suspension.
- The use of DMDM medium induced a tardily plastic attachment of bone marrow mesenchymal stem cells with a larger number of cells in suspension.
- α-MEM medium had benefic effects upon cell viability and proliferation rate detecting in the first batch a 70% confluence rate at 7 days.
- DMEM medium had positive effects upon cellular viability but with a smaller proliferation rate, detecting a 65% confluence rate at 9 days for the second batch.
- Cell viability after thawing tests detected a larger survival rate in α-MEM medium flasks.
- Comparative analyses of the two media concluded a higher efficiency rate of α-MEM medium upon cell viability and proliferation.
- Freezing and thawing determined homogenous cell morphology, typical for mesenchymal stem cells, obtaining plaques with 95 percent fibroblast-like cells.
- We recommend stem cell harvesting from bone marrow punctures as a relatively easy technique necessary for autologus and heterologus regenerative therapy.

BIBLIOGRAPHY