

INVENTION OF A MURINE BONE MARROW EXTRACTION DEVICE FOR USE IN THE DEVELOPMENT OF BIOLOGIC THERAPEUTICS FOR THE TREATMENT OF BATTLEFIELD INJURY

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INTRODUCTION

The application of cellular therapies to treat chemical injury offers a novel and promising approach to address longstanding challenges in tissue repair. Mesenchymal stem cell (MSC) treatment represents a single medical intervention that can provide a broad range of therapeutic efficacy, with local activity, at multiple tissue and organ sites simultaneously.

Intravenous (IV) MSC infusion mimics a naturally occurring process in which endogenous MSCs leave the bone marrow compartment in response to injury, enter the circulation, and travel to sites of tissue damage due to the influence of chemotactic homing signals released at each compromised site.^{1,2} Cell culture-expanded MSCs demonstrate the potential to form several specialized cell types, including skin, bone, fat, cartilage, tendon, cardiac and skeletal muscle, lung and kidney epithelium and many others. Once engrafted within damaged tissue, MSCs participate in the healing process both directly, through differentiation to replace lost cell types, and indirectly, through the local secretion of cytokines and other bioactive molecules that facilitate a reduction in inflammation, inhibition of scar formation, and the enhancement of endogenous mechanisms of tissue reconstruction.⁴

Here we report the development of a device that can be used to significantly increase the production yield of murine MSCs isolated from bone marrow. We have previously evaluated rat and rabbit MSCs as candidate therapeutics to treat injury resulting from chemical warfare agent (CWA) exposure. Extension of MSC studies to include the mouse as an experimental model will allow investigators evaluating CWA injury and treatment to take advantage of a wide array of genetic mutants available for stem cell identification, post-infusion cell tracking studies and injury pathway analyses.

METHODS I

DEVICE ASSEMBLY

The murine marrow extractor assembly is constructed using a 1.5 mL microtube, 0.6 mL microtube and 20 µl presterilized pipette tip. One prong of a fine tweezer is used to push the filter 11 mm from the large opening toward the terminus of the pipette tip (Figure 1A). The tip shaft is cut just above the filter and the tip end containing the filter is then discarded (Figure 1B). After the pipette tip and 0.6 mL microtube are cut, the pipette tip is placed into a 0.6 mL microtube (Figure 1C). The final construct is then completed by setting the pipette tip/0.6 mL microtube combination into a 1.5 mL microtube (Figure 1D). Once extractors are assembled they are placed into an autoclave safe packet and sterilized.

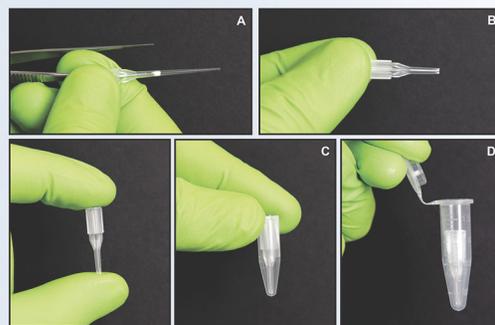


Figure 1. Assembly of the extraction device.

METHODS II

MARROW ISOLATION USING THE EXTRACTOR DEVICE

Both femurs and tibiae are used in the extractor (one bone per extractor). Prior to use of the device, the bone sample is cleaned to remove surrounding tissue. Then, 1 mm or less of bone is cut from each terminus of the tibia or femur (Figure 2A). The bone is then placed into the extractor such that the larger cavity opening is toward the device (Figure 2B). Each extractor is capped after placement of the bone to contain moisture and to minimize risk of contamination. Samples are centrifuged at 150 x g (1800 rpm in a Hermle Z 230 MR) for 90 seconds. Following centrifugation, a button-shaped pellet of bone marrow is formed that can be readily identified at the bottom of the 0.6 mL tube (Figure 2C). At this point, the pelleted marrow can be recovered for downstream operations for a single sample, or the samples can be pooled for analyses or processes requiring marrow from a large number of donor subjects (Figure 2D).

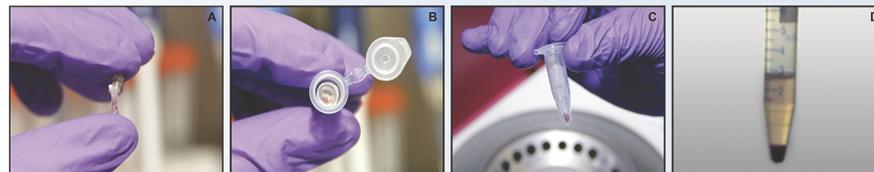


Figure 2. Preparation of the bone for marrow extraction.

RESULTS

STEM CELL GROWTH AND CHARACTERIZATION

The methods developed for murine bone marrow extraction and MSC growth described here are readily carried out in the laboratory and generate a greater volume of bone marrow source material for MSC isolation than previously reported in the literature (approximately 300 µl from the femurs and tibiae of 10 mice). The production strategy employs a unique centrifugation-based method for murine marrow isolation, maintenance of low cell culture density during growth (particularly at passage 0, as seen in Figure 3), and growth over a fibronectin-coated cell surface. Together, this approach results in the production of greater than 14 million cells of passage 3 test material within 12 days of *in vitro* culture expansion (Figure 4). Characterization of stem cell properties of isolated MSC test lots is shown by adipogenic and osteogenic differentiation (Figure 5).

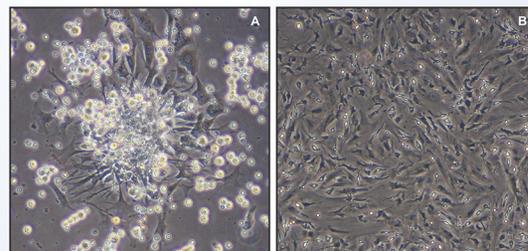


Figure 3. Isolation of MSCs from bone marrow.

One of several possible utilities of the extractor device is the isolation of bone marrow to generate purified MSC cultures. The pelleted marrow is resuspended in growth medium, plated out to fibronectin-coated tissue culture vessels and incubated at 37°C. MSCs are differentially adherent to tissue culture plastic compared to other cell types found in the bone marrow. A rinse procedure at two days post-plating and subsequent medium changes and culture passages facilitate the generation of a virtually pure MSC population by the end of P3. Figure 3A shows early colony formation during P0. Figure 3B shows the typical morphology of a mouse MSC culture allowed to reach 100% confluency.

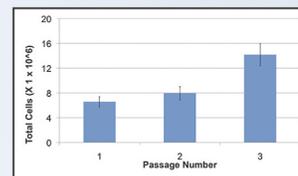


Figure 4. Murine MSC growth from Passage 1 to Passage 3.

Whole bone marrow from the femurs and tibiae of 10 mice was plated out to a fibronectin-coated T-80 tissue culture flask (P0). P0 cultures were allowed to grow until loose MSC colonies were formed. Cells were passed from P0 to P1 prior to the formation of tight cellular packing in colony centers. MSCs were then passed to new culture vessels at a density of approximately 6,000 to 8,000 cells per cm². Successive passages were carried out using identical methods once cells reached 80% to 95% confluency. Cells were harvested for cryopreservation in liquid nitrogen at the completion of P3. The results included in the graph above show growth expressed as total cell number at the end of each passage. Bars represent standard deviation.

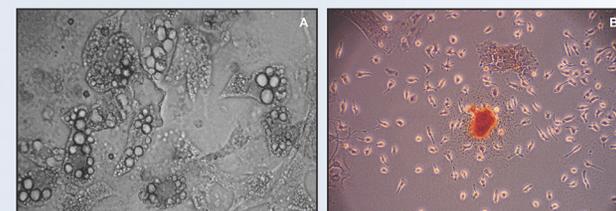


Figure 5. Demonstration of stem cell potency.

A. Cells seen here were plated out in adipogenic induction medium for 14 days. B. Cells were grown in osteogenic induction medium for 13 days. Red staining identifies areas of calcium deposition. The data in this image shows that the isolated cells are in fact stem cells as demonstrated by differentiation to form mature cell types.

CONCLUSIONS

ADVANTAGES OF THE MARROW EXTRACTOR DEVICE

- Significantly greater quantities of bone marrow from mice, compared to traditional methods. *MSCs were harvested and frozen, with a final yield of 14.2×10^6 cells.
- Reduction of contamination risk during marrow isolation.
- Significant decrease in the number of animals required to perform downstream operations.
- Reduction of cell stress during isolation procedures.
- In addition to the isolation of MSCs, the device can also be used to isolate other bone marrow constituents:
 - hematopoietic stem cells
 - mature cell types and precursors to mature cells, including lymphoid and myeloid cells
 - adipocytes, osteoblasts and their precursors
 - non-cellular bone marrow constituents, including extracellular matrix proteins, sugars and other biomolecules found in the bone marrow compartment

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