Citrate-based Anticoagulant Does Not Hinder Stem Cell Viability and Concentration from Bone Marrow Aspirate
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Materials and Methods: Aspirate Concentration – Fifty-five milliliter aspirate samples were obtained from 10 healthy volunteers (Lonza, Rockville MD) within 24 hours of harvest. Each sample was anticoagulated with 5mL of ACD-A (Citra Anticoagulant Co., Braintree, MA). Approximately 5mL was preserved to serve as bone marrow aspirates have traditionally used the anticoagulant heparin, it has been shown that long-term administration of heparin can lead to osteoporosis (2). Furthermore, therapeutic doses of heparin given during orthopedic surgery to reduce embolism formation have been shown to delay bone repair (3). To date, the effects of other anticoagulants on the function of stem cells contained in bone marrow have not been determined.

Current efforts to concentrate bone marrow progenitor cells in a rapid, point-of-care fashion employ platelet concentrate technologies that utilize citrate-based anticoagulants. Additionally, citrate-based anticoagulants are easily reversed with the addition of calcium chloride (CaCl₂), whereas the anticoagulant action of heparin is difficult to counteract. The purpose of this study was to determine if bone marrow aspirated in citrate anticoagulant could effectively be concentrated while retaining the colony-forming ability of its cells.

Materials and Methods: Ascipir Concentration – Fifty-five milliliter aspirate samples were obtained from 10 healthy volunteers (Lonza, Rockville MD) within 24 hours of harvest. Each sample was anticoagulated with 5mL of ACD-A (Citra Anticoagulant Co., Braintree, MA). Approximately 5mL was preserved to serve as base sample, while approximately 30 mL was processed using the GPSIITM Platelet Concentrate System (Biomet Biologics, Inc., Warsaw, IN)*. Following a 15-minute centrifugation cycle, approximately 6mL of a concentrated cell-rich fraction was obtained.

Analysis - Cell counts were obtained in triplicate for all base and concentrated samples using a 5-part differential hematology analyzer (Cell-Dyn 3700, Abbott, Dallas, Texas). Viability was determined using trypan blue exclusion. Colony-forming assays were performed to enumerate mesenchymal stem cells (colony-forming unit-fibroblast, CFU-F) and endothelial progenitor cells (colony-forming unit-EC, CFU-EPC). Before performing CFU assays, bone marrow samples were prepared by lysing red blood cells, followed by resuspension in PBS containing 2% fetal bovine serum. To obtain CFU-Fs, 5x10⁵ mononuclear cells (MNC) were plated into T-25 flasks containing Mesencult® media (StemCell Technologies, Vancouver, BC), and incubated for 2 weeks at 37°C and 5% CO₂. Following incubation, cultures were fixed and Giemsa stained for visualization and enumeration. Typical colonies were between 1-8 mm in diameter, and could be observed macroscopically. This macroscopic enumeration method differs from the microscopic method described in a previous report (4). For the CFU-EPC assay, 5x10⁶ MNC were plated onto 6-well fibronectin (BD Biosciences, San Diego, CA) coated plates containing EndoCult® media (StemCell Technologies) and incubated at 37°C and 5% CO₂. On day 2, all nonadherent cells were transferred to a second 6-well fibronectin coated plate and returned to the incubator. Cells were fixed and Giemsa stained on day 5. Colonies that consisted of a central core of round cells with elongated spindle-like cells radiating from the edges were counted as endothelial progenitor cell colonies.

Statistics - Data is presented as mean ± standard deviation. Statistical differences were determined using a Student’s t-test (α=0.05).

Results: Complete blood counts using a blood hematology analyzer determined the cell content in the base and concentrated cell-rich fraction samples (Table 1). The hematology analyzer categorizes the total nucleated cells (TNC) into 5 distinct white blood cell types, including neutrophils, basophils, eosinophils, lymphocytes, and monocytes. Bone marrow contains these cells, plus their progenitors, as well as mesenchymal stem cells. For this study, the cells classified as lymphocytes and monocytes were counted as mononuclear cells (MNC). It has been shown that the progenitor cells are counted in the MNC fraction (5).

The average concentration of mononuclear cells in the concentrated cell-rich fraction was 26.6 x 10⁶ cells/µL. This represented a 6.9 fold increase in concentration and a 79.7% recovery compared to the base levels. While bone marrow itself does not contain a high number of platelets (PLT), it is common to have some level of peripheral blood dilution during marrow aspiration. This is evident with approximately 1x10⁸ platelets/µL being found in the concentrated marrow samples. In addition, the percent recovery of available platelets (81.3%) is similar to reports for blood-derived platelet-rich plasma (6).

Discussion: Results from this study suggest that citrate-based anticoagulants are a viable alternative for surgeons concerned about the negative influence of heparin on bone marrow. Unlike heparin, citrate anticoagulants can easily be reversed with the addition of calcium chloride, allowing the marrow to form a coagulum and serve as a potential delivery vehicle for the contained cell population.

Bone marrow aspirated in citrate anticoagulant can be efficiently processed to increase progenitor cell concentration while maintaining cell viability. Additionally, both mesenchymal stem cells and endothelial progenitor cells maintain their ability to form colonies following concentration.

The method of concentration primarily consists of a 15-minute centrifugation process making implementation in a point of care setting feasible. Further analytical work will provide a greater understanding of the cellular population recovered with this method.

(5) Swift et al., 2007 ORS, p.1287.

Table 1. Average cell counts for marrow aspirated with ACD-A (n=10)

<table>
<thead>
<tr>
<th>Cells x10⁶/µL</th>
<th>Base</th>
<th>Concentrated Cell-rich Fraction</th>
<th>PLT Increase</th>
<th>Fold Increase</th>
<th>Colony Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNC</td>
<td>1.0±0.2</td>
<td>1.0±0.4</td>
<td>2.8±0.8</td>
<td>3.5±0.9</td>
<td>88±91.2%</td>
</tr>
<tr>
<td>MNC</td>
<td>4.4±1.1</td>
<td>20.6±3.3</td>
<td>4.9±4.7</td>
<td>7.9±7.1</td>
<td>76±51.9%</td>
</tr>
<tr>
<td>PLT</td>
<td>155±25</td>
<td>960±228</td>
<td>6.2±1.6</td>
<td>81±3.3</td>
<td>81±6.1%</td>
</tr>
</tbody>
</table>

The concentration of cells in this study was determined using a Student’s t-test (α=0.05).

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*510(k) pending, device is currently not cleared for processing bone marrow in the United States.