INSTANT STEM CELL THERAPY FOR ORTHOPEDIC APPLICATIONS: Automated separation of mesenchymal cells from bone marrow aspirates

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INTRODUCTION:
Mesenchymal stem cells (MSC) constitute an essential element in many applications in regenerative medicine. In orthopedics, they are used as osteoinductive component for bone regeneration. One source for MSC is bone marrow (BM) aspirate which contains concentrations of 10-100 MSC per Mio BM cells. This low number requires an efficient isolation procedure and usually an in-vitro expansion to increase the total number of MSC before use. Routinely, the mononuclear cell (MNC) fraction in the operating room within 1 hour. These fractions were biologically and biologically characterized and compared to MSC separated by manual Ficoll procedures and to native BM cells.

METHODS:
Bone marrow aspirates harvested from the iliac crest of haematological healthy donors (Caucasians, mean age = 24, n= 10) receiving autogenous bone grafts, were processed according to the scheme in Figure 1. The procedures were approved by the institutional ethics committee and all donors provided informed consent. 50 ml of fresh BM aspirate was divided into three aliquots: 30 ml were used for the automated separation processing (Biosafe, Switzerland) in either a Ficoll based protocol (n=5) or a volume reduction protocol (n=5). The automated Ficoll (SEPAX F) separation procedure yields in a MNC fraction while the volume reduction produced a nucleated cell (NC) fraction. The automated Ficoll procedure is divided centrifugal separation in 90 ml Ficoll-Paque® (10 min, 677 g) followed by two washing cycles with PBS containing 2.5% BSA and a final centrifugation in 90 ml PBS (10 min, 677 g). The output volume was 45-50 ml. The SEPAX volume reduction (SEPAX VR) protocol included a single centrifugation step (960 g) and resulting output volume of 8 ml. All separation protocols were automatically completed within 15 to 20 min (SEPAX F) and 30 to 45 min (SEPAX F), respectively. A manual Ficoll (Man F) separation procedure and native BM (Native) were used as control groups. The Man F procedure (n=10) included the following processing steps: washing of 15ml BM aspirate in PBS and centrifugation (10 min, 677 g), followed by re-suspension in PBS and centrifugation over 15 ml Ficoll-Paque® (30 min, 1524 g), and finally, recovering of the resulting fraction in PBS and two centrifugations (10 min, 677 g). The native BM control group was not processed and 5 ml aspirate were used directly for cell culture (n=10).

Figure 1: Experimental Design

The number of MSC was assessed by counting the CFU-F in 1 x 10^6 cells after 7 days in culture in a modified Verfaillie medium containing PDGF (10 ng/ml), EGF (10 ng/ml) and 2% FCS. Non-adherent cells were removed at a medium change after 48 hours. Fluorescence Activated Cell Sorting (FACS) analysis was performed using standard operating procedures and quantification criteria with individual settings for each marker. At passage 4, aliquots of 1 x 10^7 cells were analyzed regarding the following CD markers: CD13; CD 44; CD73; CD90; CD105; CD271; HLA-A,B,C; CD14; CD34; CD45; HLA-DR.

The capacity of the MSC to differentiate into the chondrogenic, osteogenic and adipogenic lineages was examined after passage 2 as published previously (Vogel et al. 2006). Briefly, chondrogenic differentiation was assessed after 28 days in micromass culture of initial 1 x 10^6 cells in chondrogenic differentiation medium. Dehydrated histological sections were stained with toluidine blue or antibodies for collagen type I and II. Osteogenic differentiation was assessed in monolayer cultures with cells cultured using an osteogenic media. The monolayers were fixed in 70% ethanol and stained with alizarin red-S after 14 days and quantified by incubation with cetylpyridinium chloride. Adipogenic differentiation in monolayer culture was induced by incubating in a adipogenic media. After fixation in 5% parafomaldehyde the samples were stained and counterstained with 0.3% oil-red O/60% isopropanol and hemalaun. Histological sections and cell cultures were assessed by two blinded investigators using the same semi-quantitative score rating for chondrogenic differentiation (0=no staining, 1= moderate, 2= well differentiated), and for adipogenic differentiation a rating (0=0% cells stained positive; 1=1-20%; 2=21-50%; 3=51-80%; 4=81-100%). Coloured elites of osteogenic differentiation were measured at 570 nm in ELISA reader and compared to standards prepared by diluting a 0.5% AR-S solution with CPC-solution.

The statistical effect of the MSC isolation protocol was examined by multifactorial analysis of variance (ANOVA). The alpha error was consequently adjusted; p values <0.05 were considered significant. All tests were two-tailed.

RESULTS and DISCUSSION:
All groups differentiated into the three tested mesenchymal lineages. Assessment of the chondrogenic and adipogenic differentiation revealed no significant differences even though, the Native group seemed inferior in chondrogenic analysis as compared to the other groups. Chondrogenic differentiation scored 24 point of 34 points maximum for SEPAX F, 15 of 34 for Man F, and 10 of 38 for Native. Osteogenic analysis resulted in a high donor to donor variability, but there was no difference between the groups. Quantification of the CFU-F/ml was highest with 228 ± 107 for the Sepax VR and 155 ± 98 for the Native groups followed by Sepax F (86 ± 46, p=0.0042) and Man F (50 ± 36, p=0.001). FACS analysis resulted in a near identical CD pattern for all groups with similar percentages of positive/negative cells. The following characteristic were found: CD 13+; CD 44+; CD73+; CD90+; CD105+; HLA-A,B,C++; CD14++; CD34++; CD45++; CD271++; HLA-DR++.

Instant stem cell therapy has been described on several occasions and is mainly under clinical investigation for cardiac and orthopedic applications. It is essential for clinical use that a sufficient number of MSC can be used instantly for therapy without the need for in vitro expansion. Therefore a highly efficient method has to be found for isolation of MSC. Our study demonstrates that the SEPAX separation system is capable to separate MNC and NC fractions at the same quality as native BM cells or obtained by manual separation procedures. The yield was, however, higher in the automated SEPAX sequences. In our experimental setting, the SEPAX VR protocol resulted in the best yield and highest content of cells MSC. On going studies show that by optimizing the sequences and input volumes, the number of MSC per million cells can be increased by a factor up to 1.4 in the extract resulting in a sufficient number of cells required for therapy.