Introduction: Bone marrow derived mesenchymal stem cells (MSCs) are commonly obtained via adhesion and proliferation on tissue culture plastic. While cell-surface markers have shown homogeneity in these populations [1], we have found that adult equine MSCs do not undergo uniform chondrogenesis as a significant portion of cells die over time in chondrogenic culture [2]. In this study, we investigated the chondrogenic potential of cells that survive the initial stages of chondrogenic culture in a scaffold-free suspension culture. This subpopulation was compared to normal preparations of MSCs to determine whether chondrogenic differentiation could be increased using this selection technique.

Methods: Tissue harvest, cell preparation, and encapsulation in agarose – Bone marrow was harvested from 5-8yr old horses. The nucleated cells were seeded in low glucose DMEM + 10% FBS at a concentration of 0.25x10^6 cells/cm^2. Adherent MSC colonies that formed after 6-8 days were further expanded by reseeding in AlphaMEM + 10% FBS + 1 ng/ml FGF-2 [3] at a concentration of 1x10^5 cells/cm^2. Each MSC population was then cryopreserved (95% FBS, 5% DMSO). Experiments were initialized by thawing and plating in AlphaMEM expansion medium through one passage. A portion of the MSCs were cryopreserved, while 1x10^6 MSCs were seeded into suspension cultures created with a polyHEMA-coated 775 flask in defined medium (ITS+, 0.1 μM dexamethasone, and 37.5 μg/ml ascorbate-2-phosphate) plus 10 ng/ml TGFβ-3 (R&D Systems) [4]. After 3 days, the MSCs in the suspension cultures were trypsinized to create an individual cell suspension, which was seeded for expansion. Cryopreserved MSCs that had not been cultured in suspension were thawed and expanded in parallel. After 2 days, both cultures were seeded in 2% agarose at a concentration of 10x10^6 cells/ml and cultured in defined medium +/- 10 ng/ml TGFβ-3, creating ‘Suspension’ and ‘Monolayer’ groups in the absence (TGF-) or presence (TGF+) of TGFβ-3. Separate experiments were run for each donor animal. Analysis - Cell viability was analyzed using a calcein (live)/ethidium bromide (dead) viability kit (Molecular Probes). Total GAG accumulation in the scaffold was quantified using the DMDB dye binding assay. Over the final 24 hours, samples were evaluated for protein and proteoglycan synthesis via [3H]-proline and [35S]-sulfate radiolabel incorporation, respectively. Proteoglycan staining was colocalized with the viable cell population by incubating calcein-labeled samples in a 0.0005% Toluidine blue solution. Statistics - Mixed model analysis of variance with individual comparisons using least square means procedure. p-values>0.05 were considered significant.

Results: Suspension cultures resulted in the formation of MSC clusters containing 10s to 100s of cells. Recovery of viable MSCs from suspension clusters ranged from 34-49%. Subsequent monolayer expansion resulted in 2.6-4.6-fold increases in cell number after 2 days.

Cell viability in agarose: All cultures were greater than 95% viable immediately after casting. In Monolayer TGF-, viability decreased to ~27% over 15 days (Fig. 1). In Suspension TGF-, viability was greater than Monolayer TGF- at each timepoint (p<0.01), with a viability of ~58% after 15 days. In Monolayer TGF+, viability was greater than Monolayer TGF- (p<0.01) but less than Suspension TGF- (p<0.01) through day 12. On day 15, Monolayer TGF+ viability was ~50%, higher than Monolayer TGF- (p<0.01) and similar to Suspension TGF+ (p=0.18). Viability in Suspension TGF+ was higher than all other conditions at each timepoint (p<0.01), with a final viability of ~67%.

Extracellular matrix (ECM) synthesis: For TGF- cultures, biosynthesis in Suspension samples was 4.8-9.2-fold higher than Monolayer (p<0.005) (Fig. 2). For TGF+ cultures, biosynthesis was similar between Suspension and Monolayer groups (p=0.61-0.81). Relative to Monolayer TGF+, biosynthesis in Monolayer TGF- was less than 7% (p<0.005), while Suspension TGF+ was 25-55% (p<0.005). Toluidine blue staining: In Monolayer TGF-, almost no toluidine blue staining was detected (data not shown). In rare instances an MSC was surrounded by toluidine blue-positive matrix. In Suspension TGF-, toluidine blue positive cells were more numerous (Fig. 3a). However, proteoglycans had not accumulated around many viable cells (Fig. 3b, calcein image for 3a). In both TGF+ cultures, the majority of the viable cell population was surrounded by proteoglycan matrix, with few viable cells showing no toluidine blue staining (data not shown). Subjectively, the average area of proteoglycan staining around positive cells in TGF+ cultures appeared similar to the Suspension TGF- image in Fig. 3A.

Discussion: Similar to agarose cultures, suspension cultures of clustered MSCs reduced the number of viable cells. In subsequent agarose cultures, suspension cells survived better than did monolayer cells for a given TGF condition. These data suggest that the suspension culture result in an MSC population that may better tolerate potentially challenging environments in vivo.

As seen previously, Monolayer MSCs maintained in the absence of TGF synthesized little ECM [5]. However, the presence of toluidine blue staining around a very small subset of cells suggests that ECM synthesis was not uniform over the viable cell population. In Suspension TGF-, MSCs surrounded by proteoglycan matrix were more frequently interspersed among viable cells that did not stain for toluidine blue. Furthermore, the highest level of ECM synthesis found in TGF+ cultures coincided with the greatest frequency of toluidine blue positive cells. These results suggest that overall ECM synthesis is at least partially dependent on the frequency of differentiation into a highly active chondrocyte-like phenotype. In the absence of TGF, suspension conditioning was sufficient to select for chondrogenic MSCs and/or induced differentiation to a highly active phenotype in a subset of cells. These results demonstrated a benefit of suspension conditioning for environments where chondrogenic stimuli are minimal.

In most cases, MSCs surrounding a proteoglycan matrix were more frequently viable in suspension with suspension conditioning did not translate into higher ECM synthesis. Therefore, these data suggest that suspension conditioning offers little to no benefit in highly chondrogenic conditions. Based on the high frequency of toluidine blue-positive cells in TGF+, it is possible that the lack of effect with cluster conditioning may be attributed to the effectiveness in continuous treatment of TGF+ to induce more uniform differentiation. Furthermore, the ability to pre-differentiate MSCs into a chondrocyte-like phenotype prior to implantation is anticipated to increase the cartilage repair potential over the use of undifferentiated MSCs. The significant increase in ECM synthesis with cluster conditioning in TGF+ medium demonstrated that cluster conditioning was sufficient to increase chondrogenesis, although to a lesser extent than longer exposure to chondrogenic medium. Our method was developed to obtain an individual cell suspension following differentiation culture. Therefore, MSCs may be prepared for applications where culture of a cell-seeded construct is not desired, such as in situ scaffold casting or joint injections. Furthermore, this study demonstrated that chondrogenic differentiation was not fully reversed by subsequent expansion culture.