The Effects of Fibrin Gel on Viability of Bone Marrow Stem Cells

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Introduction: Cell based tissue engineering techniques have been introduced recently, with encouraging outcomes in both in vitro and in vivo models. Stem and stromal cell treatments to the injured tendon require a delivery vehicle, such as collagen, platelet rich plasma, or fibrin. A number of studies have investigated the effect of fibrin scaffolds with stem cells, with and without growth factor augmentation(1-4). Results have not been consistent, with observations made that fibrin scaffolds increased cell viability, proliferation, or differentiation and that it may adversely affect these parameters. Fibrin gel, acting as a carrier of GDF-5 treated bone marrow stem cells (BMSCs) and muscle derived stem cells (MDSCs), enhanced tendon healing, evaluated both mechanically and histologically, compared to a collagen gel in an in vitro tendon repair model at 2 weeks and 4 weeks. However, the concentration of fibrinogen and thrombin used in this study was based on levels necessary for hemostasis. Fibrin is also used as a tissue repair glue, but at much higher concentrations of both fibrinogen and thrombin, and the degree of adhesion of the resulting fibrin depends on the concentrations of these compounds.

The purpose of this study was to investigate the viability of BMSCs seeded in fibrin patches created from various concentrations of fibrinogen and thrombin in culture for one, three, and seven days. We hypothesized that fibrin concentration would affect cell survival and mobility.

Methods: A mixed-breed dogs euthanized for other Institutional Animal Care and Use Committee (IACUC) approved studies were used to harvest the bone marrow. The fibrin gel was divided into five groups differing in the formulation of the fibrin produced, the formulations having been derived from previous studies: a) 5mg/mL fibrinogen and 25 U/mL thrombin (hemostatic concentration group) b) low adhesive concentration fibrinogen (40 mg/mL) and low adhesive concentration thrombin (250 U/mL) interposition (LL group), c) high adhesive concentration fibrinogen (80 mg/mL) and low adhesive concentration thrombin (250 U/mL) interposition (HL group), d) low adhesive concentration fibrinogen (40 mg/mL) and high adhesive concentration thrombin (500 U/mL) interposition (LH group), e) high adhesive concentration fibrinogen (80 mg/mL) and high adhesive concentration thrombin (500 U/mL) interposition (HH group). Bone marrow fluid was collected, under sterile conditions, from each tibia. Cells were pelleted and supernatant fluid was removed by centrifugation at 1500 rpm for 5 minutes at room temperature. The bone marrow pellet was resuspended in cell culture medium (MEM) with Earl’s salts (GIBCO, Grand Island, NY, USA), 10% fetal bovine serum and 1% antibiotics (Antibiotic-Antimycotic, GIBCO, Grand Island, NY, USA) and then plated onto two 100-mm culture dishes in 10 mL of MEM. Bone marrow cells were incubated at 37ºC with 5% CO2 and 95% air at 100% humidity. BMSCs of passages 2 were used for seeding into the fibrin gel. For the seeding, 1.3 X 105 cells were pelleted down. Each group has three patch which was fabricated from a mixture of a 20 μL aliquot of bovine fibrinogen (Sigma-Aldrich, St. Louis, MO, USA) with the pelleted BMSCs added to a 6 μL bovine thrombin solution
(Sigma-Aldrich, St. Louis, MO, USA), each constituent having the respective concentrations described above. The gel was incubated at 37ºC for 0.5 hours to complete gelation. The fibrin patch was then dispensed onto a 24 well plate with MEM and Earle’s salts (GIBCO), 10% fetal bovine serum and 1% antibiotics (Antibiotic-Antimycotic, GIBCO), and incubated at 37ºC in a 5% CO2 humidified incubator for one, three, or seven days. Culture medium was changed every other day. After one, three, or seven days in culture, cells were incubated with calcein AM and ethidium homodimer-1 fluorescent dye (EthD) solution (Live/Dead® viability/ cytotoxicity kit for mammalian cells; Life Technologies, Grand Island, NY, USA) to stain for living and dead cells. Stained specimens were examined with a laser scanning confocal microscope (LSM780; Zeiss, Germany) for live-dead counting and to examine cell morphology. Living and dead cells were counted using ImageJ software (National Institute of Health, Bethesda, MD, USA), and results reported as a the mean count of living cells on 10X-field and the ratio of dead cells to total cells (living and dead) counted at day 1, 3, and 7. One dish from each group was also prepared for a scratch assay to assess cell migration. In this test, a p-200 pipette tip was used to score (300-500 μm width) the fibrin in each dish. Then they were observed once a day until day 7.

Statistical Analysis: Difference of mean count of living cell on 10X-field and the ratio of dead cells to total cells among each time and group were analyzed by two-way factorial analysis of variance (ANOVA). The Tukey-Kramer post hoc test for each pairwise comparison was performed if there was a significant difference. The significance level was set to p<0.05 in all cases.

Results: Two types of cell morphology were observed: round or spindles with processes (Fig. 1). The mean count of living cells on 10X-field at {day 1, day 3, day 7} were {136, 196, 77}, {192, 358, 247}, {432, 361, 203}, {419, 418, 255} and {476, 235, 216} for the control, LL, LH, HL, and HH groups, respectively (Fig. 2). Two-way factorial ANOVA results indicated that mean count of living cell in control group is smaller than the LH, HL, and HH group significantly (P < 0.005). The ratios of dead to total cells at {day 1, day 3, day 7} were {0.17, 0.03, 0.11}, {0.16, 0.17, 0.14}, {0.10, 0.16, 0.18}, {0.13, 0, 0.07} and {0.10, 0.01, 0.03} for the control, LL, LH, HL, and HH groups, respectively. Two-way factorial ANOVA results indicated no significant difference among the group. The scratch assay showed cells filling the gaps at one day in control, LL, and LH groups, and at seven days for the HL group. There was no cell migration in the HH group (Fig. 3).
Fig. 1  

Immunohistochemistry

Morphology

Day 3

Control  LL  LH  HL  HH

Spindle  Spindle  Spindle  Round  Round - spindle

Day 7

Control  LL  LH  HL  HH

Spindle  Spindle  Spindle  Round  Round - spindle

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Fig. 2

Mean count of Living cell (on 10X-field)

Dead cell,total cell

Two-way factorial ANOVA

Interaction: N.S.

* P<0.05

** P<0.016

The error bars represent the standard deviation of the measurements
**Discussion:** Cell migration potency may be extremely important for tendon healing. In this study, the high fibrinogen groups had little migration potency, and the morphological findings showed that the BMSCs in the HL and HH groups maintained a rounded shape. These finding suggest that high concentrations of fibrinogen impair cell migration, and this may impair tendon healing. On the other hand, mean count of living cell in control group was lower than the other group. Thus we conclude that, if used in the context of tendon repair, lower adhesive concentrations of fibrinogen should be used.

**Significance:** We conclude that it is important to optimize fibrinogen and thrombin concentrations when using a cell-seeded fibrin patch in tendon repair settings.

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