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An in-vitro study to determine the feasibility of combining Bone Marrow Concentrate with BST-Cargel as a treatment for cartilage repair

1. Introduction

The microfracture (MF) technique represents a first-line treatment option for small to medium size full-thickness cartilage defects in the knee, showing a decrease in pain and improved knee function after 1- or 2-year follow-up[1–3]. In marrow-stimulating procedures, perforations to the subchondral bone allow blood-marrow ~~and marrow-~~ derived cells to migrate into the holes and defect forming a blood clot. The subsequent wound repair cascade comprised of an acute inflammatory response and cell chemotaxis leads to the generation of a vascularized granulation tissue, and the potential proliferation of pluripotent mesenchymal progenitor cells with a capacity to differentiate into multiple mesenchymal cell types[4]. Bone remodeling can proceed along with the induction of chondrogenesis, that resembles endochondral ossification, to form new bone in deeper zones and fibrous or fibrocartilaginous tissues in the more superficial chondral region[1,5,6].

However, longer term follow-up has demonstrated increasing failure over time with the MF technique[7,8] particularly in lesions >4cm^2 [9]. As a consequence, clinicians and researchers have tried to improve MF results by augmenting the blood clot with various scaffolds, a treatment termed Autologous Matrix Induced Chondrogenesis (AMIC). The quality of the data regarding the results for AMIC is currently insufficient to recommend one scaffold over another with few prospective studies comparing the results of AMIC to MF or Autologous Chondrocyte Implantation[10].

BST-CarGel® (~~Piramal Life Sciences~~Smith & Nephew, ~~Bio-Orthopaedic Division~~Andover, Massachusetts) was developed by Hoemann et al. to stabilize the blood clot in the cartilage lesion following microfracture by dispersing a soluble polymer scaffold containing chitosan throughout the blood[11,12]. Chitosan is an abundant glucosamine polysaccharide derived from the exoskeleton of crustaceans with low toxicity along with good biocompatibility, biodegradability, and tissue adhesive properties[13]. By dissolving chitosan in an aqueous glycerophosphate buffer[11], BST-CarGel® is obtained as a liquid chitosan solution having physiological pH. When mixed with fresh, autologous whole blood, BST-CarGel® does not interfere with normal coagulation, but reinforces the resulting clot by impeding its retraction[14]. The increased clot adhesivity within the lesion as a consequence of chitosan's cationic nature ensures prolonged activation of tissue repair processes by maintaining critical blood components above the marrow access holes[12,14,15].

An 80 patient prospective randomised controlled trial of BST-CarGel® versus microfracture for the treatment of isolated full-thickness lesions in the knee resulted in more voluminous, higher quality cartilage repair at 1 year[13]. The benefit was sustained over 5 years, supporting the hypothesis that BST-CarGel® increases the consistency of cartilage repair and enhances long-term structural superiority compared with MFX[16]. The study was not powered for clinical outcome and no significant difference in clinical scores was seen between groups.

The potential flaw in the AMIC process is the continued need for the microfracture to provide a source of pluripotent cells in order to induce repair. The bone changes induced by the microfracture potentially will compromise the long-term results. Bone overgrowth following micro fracture has been reported in up to 80% of cases[17], with its presence associated with a ten times increased rate of failure[18]. In an attempt to avoid microfracture researchers have explored the potential of using bone marrow concentrate (BMAC) as an easily accessible source of pluripotent cells, with promising results reported in the literature[19,20]. There is little data within the literature regarding the most effective scaffold/BMAC combination with previous work suggesting cell retention as a potential issue with textile-based scaffolds[21].

This study aimed to determine whether BST-CarGel® can be mixed with BMAC to create a cell seeded implant with comparative properties to standard BST-CarGel® mixed with blood. We also aimed to confirm cell retention and viability within the BST-CarGel®/BMAC clots.

2. Methods

Ethical approval was obtained to perform the study from the regional ethical committee reference 12/EE/0136. Twelve patients undergoing cartilage repair surgery using BMAC were consented to take part. The *in vitro* testing model used was a validated model developed by Pira~~i~~mal and one used to undertake quality assurance testing of the chitosan product. Six conditions were tested: 1. BST-CarGel® mixed with Whole Blood (CG-WB) 2. BST-CarGel® mixed with Bone Marrow (CH-BM) 3. BST-CarGel® mixed with Bone marrow concentrate (CG-BMAC) 4. Whole Blood (WB) 5. Bone marrow (BM) 6. Bone marrow concentrate and batroxobin (Plateltex®, Prague, Czech Republic)~~ibin~~ (BMAC-BTX). Figure 1.

For each patient 10 mLs of blood was taken at the time of anesthesia induction. Bone marrow collection was then undertaken from the anterior iliac crest by the senior author (MS) using a Jamshidi needle pre-rinsed with 1:1000 heparin. 60mL of bone marrow was then withdrawn into 6 x 10mL syringes pre-filled with 1 mL of

1:1000 heparin solution. Finally, 10 mL of bone marrow was withdrawn into an empty 10 mL syringe. The Harvest®, SmartPrep2® system (Smart PRePVR, Harvest Technologies, Plymouth, MA) was used to prepare the BMAC as this was found to provide the optimum BM to plasma ratio on preliminary testing. The 60mL of anti-coagulated bone marrow was first transferred into the MarrowPREP filter bag (to remove bone spicules) and then the filtered marrow was transferred into the SmartPrep®2 container, for centrifugation as per device instructions. After centrifugation, approximately 6mL of BMAC was obtained on average. BST-CarGel® reconstituted product was prepared as per the instructions of use prior to mixing with blood, bone marrow (BM) and bone marrow concentrate (BMAC). Batroxobin enzyme (BTX, Plateltex®, Prague, Czech Republic) was used as external clotting agent to re-activate the BMAC (which contained anti-coagulant) specimen. Lyophilized BTX (5 U) was re-suspended with 0.5mL of 10% Calcium Gluconate (CaG). Once BST-CarGel® and BMAC was mixed, BTX was added immediately before dispensing these mixtures into glass tubes.

For each of the test conditions, approximately 350µL of mixture was placed in a glass test tube seated in a water bath at 37°C for 60 minutes. The time taken for a half sphere clot to form at the base of the test tube was recorded. The % clot retraction and macroscopic structure of each generated clot was evaluated at 60 minutes. Three test tubes were used for each test condition. The BST-CarGel®/specimen clot samples should be "clotted" or "clotted on the edges, with a fragile or liquid" centre within 15 min to be considered acceptable and comparable to BST-CarGel®/blood clots.

Percentage of clot retraction was evaluated based on the volume of serum extruded from the clot in the test tubes during contraction. After the 60 minute clotting period at 37°C, the glass tubes were removed from the water-bath and their weight individually (including the clot and serum) recorded. All liquid (serum or un-clotted blood) extruded from the clot was carefully removed using a 1mL syringe with an 18G 1½ needle without disrupting the clot. The tube with only the clot inside was then weighed. The % of clot retraction was calculated as the % ratio of difference in weights between the clot samples with and without serum using the following formula (Equation 1):

$$\%\ clot\ retraction = 100 - \left(\frac{Mass\ of\ clot\ without\ serum(g) - Mass\ of\ empty\ tube(g)}{Mass\ of\ clot\ with\ serum(g) - Mass\ of\ empty\ tube(g)} \times 100\right)$$

Equation 1

The clots without serum from the % clot retraction test were used to assess macroscopic appearance in all groups. The clot color, firmness and shape along with the presence of fragmentation were documented.

Histological analysis

Toluidine blue staining method was chosen to evaluate clot homogeneity as it specifically stains each component throughout the clot: - Erythrocytes: blue (blue-green); - White blood cells: dark purple; - Chitosan: light blue. Briefly, blood clots were fixed in 10% Neutral Buffered Formalin and then cut into 2 halves through the middle and the cut face of the clot was placed face down into the embedding block. The paraffin blocks were then sectioned into 5µm slices and mounted on glass slides. Two slides were prepared for each sample. These slides were then stained following the toluidine blue staining method and digitally scanned and viewed at magnifications up to 40X to evaluate the clot mixing homogeneity. The criteria for acceptable histology results are defined in table 1.

Cell culture

Clots were cultured in the glass tubes in 2mL of chondrogenic or non-chondrogenic media which was refreshed every 2 days. All clots were stored in a cell culture incubator at 37°C and 5% CO_2.

Chondrogenic media:

DMEM/F-12 (50%/50% vol. mix, Gibco 21331-046, Life Technologies, UK) with 1%vol, ITS-X (Gibco 51500-056, Life Technologies, UK), 2.4%vol HEPES Buffer (H0887, Sigma-Aldrich, UK), 2.4%vol L-Glutamine (G7513, Sigma-Aldrich, UK), 1%vol Penicillin/Streptomycin (P4333-20ML Sigma-Aldrich, UK), 100nM Dexamethasone (Sigma-Aldrich, UK) , 27.5ug/mL Ascorbic Acid (2-phospho-L-ascorbic acid (49752-10G, Sigma-Aldrich, UK) 1.25ug/mL Bovine Serum Albumin (A8806-1G, Sigma-Aldrich, UK) and 10ng/mL TGF-β1 (PHG9214, Life Technologies).

Live-Dead staining

Clots were transferred from the glass test tubes to 35mm live cell imaging dishes (CELLview 627860, Greiner Bio-One, UK). Calcein AM stock solution of 4μM was prepared in anhydrous DMSO, while a propidium Iodide stock solution of 2mM was prepared with a 1:4 v/v mix of DMSO/ultrapure water. A working live/dead staining solution was then prepared by adding 0.5μL of stock Calcein AM and 2μL of stock propidium to every 1mL DMEM/F-12 culture media (2mL of live/dead working solution is required per clot). Samples were stained by adding 2mL of the live/dead working solution to the culture dish containing the clot and incubating for 15 minutes in a cell culture incubator at 37°C and 5% CO_2.

Confocal fluorescence imaging

Zeiss LSM 710 confocal fluorescence unit attached to a Zeiss Observer.Z1 microscope with a heated stage (Carl Zeiss Ltd., UK). Live and dead dyes were excited and the fluorescence collected independently as follows - calcein AM: excited with 488nm laser, ~3% transmission, spectral detection range: 500nm-592nm. Propidium iodide: excited with 543nm laser, ~7% transmission, spectral detection range: 595nm-704nm. Four frames were collected per channel and averaged to form a final image slice for each channel. Images were acquired at 1024x1024 pixels and 16-bit colour depth.

A global view of the clot was obtained by acquiring a 2D single image slice with 12μm slice thickness using a x10 (EC Plan-Neofluar 10x/0.30 M27) objective lens. Higher magnification 3D z-stacks were acquired using a x20 (EC PlanN NA=0.5 DIC) objective lens.

3. Results

The results of the blood, bone marrow and BMAC analysis pre and post centrifugation is shown in table 2. The white cell concentration on average increased by a factor of 6.9 following centrifugation of the bone marrow.

The macroscopic appearance of the BST-Cargel clots were all very similar. The clots in all BST-CG groups were classified as a pass at the 15 minute observation time point (or less). However, 2 out of 12 bone marrow specimens were not fully clotted within 15 minutes. This may be attributable to the fact that these particular samples might have been contaminated with whole blood, which would have increased the coagulation time by dilution. Indeed, it is usual to observe that the whole blood condition is not entirely clotted after 15 minutes. On the other hand, the use of batroxobin accelerated the clotting of CG/BMAC

samples and reduced the clotting time to approximately 5 minutes on average. The CG-BMAC clots were softer on manipulation and handling compared to the CG-WB and CG-BM groups.

The clot retraction for all test conditions are shown in Table 3. The CGWB samples demonstrated 2.6% retraction, which is standard retraction for the BST-CG product. CG-BM demonstrated very similar contraction however the CG-BMAC samples had a mean contraction of 10.3%.

A summary of the data on clot morphology and composition for a single patient is shown in Figure 2. CG-WB clots exhibited the typical uniform distribution of chitosan without any chitosan aggregates. Most Red blood cells had the typical biconcave morphology with small areas of agglutination. The CG-BM clots showed similar appearances with all clots classified as a pass.

The CG-BMAC clots demonstrated mostly homogenous distribution of chitosan throughout the clot and the RBCs were biconcave in shape with small areas of agglutination. However, there was variation observed in this group. 8/24 clots examined demonstrated roundish/swollen RBCs without the typical biconcave morphology. The space around the RBC's was filled up with a light blue "film" of uncertain origin. A high number of other cell types were visible as well as adipocytes. Figure 3.

Cell viability

The clots started to fragment in culture between day 3-5, leading to loss of mostly live cells adhered to the gel material during media changes. As can be seen over the image timepoints in Figure 4 c-e, the cell density decreased across the clot fragments, which reduced the total cell count and increased the range in estimated cell viability over time. The average viability dropped to 70-73% at day 3-7 due to the loss of cells attached to broken clot fragments, but homogenization of the remaining clot and clot fragments to recover the entire cell population showed that the overall viability was still >86%.

4. Discussion

The main findings of this study were that BM and BMAC (processed using the Harvest system and reactivated with batroxobin) when combined with BST cargel produced a product that had similar clot retraction, macroscopic properties and histological appearance to standard BST-CarGel® mixed with blood.

Mononucleated cells from the BMAC were retained within the scaffold and remained viable until clot dissolution *in vitro*.

Microfracture results in bone overgrowth which reduces the volume of the cartilage regenerate[18]. The subchondral bone is also postulated to become stiffer which results in increased stress on the fibrocartilage regenerate. These are thought to be some of the reasons in association with poor clot stability[14] why microfracture results appear to be less durable than those for ACI - a technique which does not disrupt the subchondral bone. The application of BMAC to chondral defects has shown promising results in both animal models[22] and in humans[20,23,24]. The aim is to provide a source of mono-nucleated cells which could augment and drive host repair and consequently this would negate the need to penetrate the subchondral bone. However, there is very little literature to guide the decision as to which is the ideal scaffold to deliver and retain the cells within a chondral defect.

Bone marrow from the iliac crest has been shown by De Girolamo et al. to contain more cells with an MSC phenotype (CD34−/CD45low/ CD271high) (0.04%) compared to the subchondral location of the defect[25] (0.02%) and so potentially its application in a chondral defect would result in increased MSC concentration compared to that provided by microfracture. By centrifuging the BM to create BMAC the mononucleated cell concentration can be increased further by a factor of 6. However, the process of centrifugation requires anticoagulation which affects the normal clotting process that BST-cargel requires to gelate. Batroxabin is a thrombin-like enzyme of Bothrops atrox moojeni venom, it specifically cleaves fibrinogen alpha chain, resulting in the formation of non-crosslinked fibrin clots. In preliminary testing this enzyme was found to be the most effective method of reactivating the BMAC without causing excessive contraction. This is believed to be due to its mechanism of coagulation via fibrin and not platelet activation[26,27]. Batroxobin has been used in clinical trials aiming to reduce perioperative blood loss in spinal surgery[28] and to prevent restenosis/reocclusion following angioplasty[29].

The main aim of the study was to replicate the macroscopic and histological appearance of the standard BST Cargel using BMAC instead of whole blood, given the scaffolds proven effectiveness to improve the quality of the cartilage regenerate in both an animal model and in humans. Hoemann et al. (Hoemann et al. 2005b) demonstrated in a sheep model that the application of a chitosan-glycerol phosphate/blood clot to condylar defects resulted in a net increase in fill (52%), a modest but significant increase in hyaline character (86%) with more columnar organization and a complete restoration of normal glycosaminoglycan levels compared to microfracture alone[14]. Stanish et al retrieved 38 repair tissue biopsies at 1 year post-treatment

as part of a RCT of BST-CarGel® versus microfracture alone in humans[13]. They found significantly better zonal organization and collagen characteristics for the BST- CarGel® biopsies over the MFX biopsies. Whilst there was no clinical difference detected between the two groups at 12 months, it is recognised that improved collagen content and zonal organization are necessary components for long-term durability of repair cartilage given collagen breakdown is considered to be a critical step in the progression of osteoarthritis.

The aim of BST-cargel is to stabilise the clot produced by microfracture and prevent clot contraction within the defect. Bone marrow contains less platelets (as platelets are only released into the circulation through the cytoplasmic fragmentation of megakaryocytes cells) and less fibrinogen (as this protein is secreted by hepatocyte cells into the circulation) than blood. However, the percentage clot retraction of BST-CarGel®/BM in this study was within the normal range of percentage clot retraction observed for BST- CarGel®/blood clots. These results are likely attributable to the fact that during aspiration of BM, venous blood was also aspirated. Therefore, BM is never 100% composed of bone marrow elements, but is also "contaminated" with venous blood. In this experiment, bone marrow elements were observed on histology (i.e. adipocytes cells and precursor's cells surrounded with collagen structures) and therefore the BM specimen collected was effectively composed of bone marrow and not only of blood. CG/BMAC demonstrated contraction on average of 10% which we feel is within an acceptable clinical range. This increased contraction is likely due to the increased platelet activation and augmented clotting process. This would be in agreement with the reduced clotting time of less than 5 minutes in this group compared to the 15minutes in whole blood control.

Macroscopically, the CG-BMAC clots were softer compared to the CG clots generated using blood and bone marrow which is likely due to the increased cellularity of the clots. The structural properties of the cargel clots were not objectively compared by undertaking mechanical testing and therefore were not objectively quantified during this study . The senior author (MS) has implanted CG/BMAC in vivo and the physical properties were comparable to CG-WB and appear to be sufficient to allow stable implantation.

The histology of the BST-cargel/BMAC clots were all satisfactory and on the whole showed similar structure to the BST/WB clots. There was even distribution of chitosan with biconcave structure of the rbc's in the majority of clots. There was variation in the CG-BMAC clots, with 36% of clots demonstrating a number of blue patches. We believe these represent de-granulated platelet aggregates, fibrin, and chitosan with no red blood cells. This was likely secondary to the heparinized blood, as calcium is not chelated, it permits platelet

aggregation and partial platelet activation following the addition of batroxabin. Whether the presence of this variation would affect the scaffold performance and the clinical outcome is unknown.

The viability data demonstrated that the cells remained viable until clot disruption. The poly-cationic nature of chitosan facilitates adhesion to negatively charged surfaces of tissues and cells[15], including cartilage. This results in increased clot adhesivity within the lesion and contributes to cell retention. This will potentially result in prolonged activation of the tissue repair processes by maintaining greater numbers of mononucleated cells within the lesion. The importance of cell retention at the defect site has been demonstrated by Kim et al., who demonstrated that implantation of MSC's in a chondral defect was superior compared to injection, probably secondary to more effective delivery of paracrine factors and less cell death[30].

Whilst a number of investigators have assessed the growth and chondrogenic potential of bone marrow derived mesenchymal stem cells on different scaffolds[31–35] there are very few studies which have undertaken the same using BMAC. Grigolo et al. seeded BMAC on a nano-structured bio-mimetic three-layer gradient scaffold composed of type I collagen and magnesium enriched hydroxyapatite[36]. They were able to demonstrate that BMAC could differentiate along chondrogenic and osteogenic pathways as evaluated by the expression and production of specific matrix molecules. Cavallo et al. seeded 0.1ml of BMAC on a 0.5cm x 0.5cm pieces of hyaluronic scaffold (Hyaff 11) and cultured them for 52 days[37]. They demonstrated that cells within BMAC seeded onto the Hyaff-11 scaffold and were able to differentiate down a chondrogenic pathway, expressing and producing sulfated glycosaminoglycans, SOX-9, aggrecan, and collagen type II. However, this is in contradiction to the findings of Kohli et al. who seeded 30µL of BMAC on Chondro-Gide®, Alpha Chondro Shield® and Hyalofast®[21]. All cell-seeded scaffolds were fed with standard culture media, three times a week for a period of four weeks. Alpha Chondro Shield and Hyalofast demonstrated the best initial cell seeding with BMAC on all three scaffolds remaining viable after 1 day and 1 week. Very few viable cells were visualised after 7 days and no cells were seen to be incorporated in either of the three scaffolds after 4 weeks in culture with cell retention postulated as the main cause.

The viability tests in this study were only carried out to 7 days. This was secondary to technical difficulties due to rapid clot degradation in vitro. BST-CarGel® degrades *in vivo* over a period of 5 weeks, however the clots in all test groups began to lose structural integrity after 7-10 days in media. We hypothesised the fragmentation occurred due to a combination regular media replacement gradually breaking its adherence to culture surfaces (despite best efforts to be careful) and loss of 3D structural support when the clots had to be

transferred to imaging dishes to be examined. Culturing the clots in imaging dishes reduced the impact of damage from moving the clots but came at the expense of structural support compared to the test tube. We also believe that the decreased viscosity of the media compared to synovial fluid penetrated the clots to a greater extent resulting in their early disruption. The increased fragility over time meant it became technically difficult to avoid scaffold and cell loss and consequently a decision was made not to image beyond the 7-day period.

The results of this study confirm that BMAC can be combined with BST cargel when prepared in a specific manner to produce a scaffold which has similar properties to standard BST cargel mixed with blood. BST-cargel has previously been shown to be a biologically active scaffold, improving histological repair following microfracture. By combining BST-CarGel® with BMAC in the manner described, bone marrow derived mononucleated cells can be retained within the chondral defect potentially negating the need for microfracture. Further *in vivo* work is required to confirm these potential benefits and determine if this combination will result in more durable cartilage repair and improved clinical outcomes.

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Figure legends

Figure 1. Schematic presentation of the clot variants studied. BMAC: Bone Marrow Aspirate Concentrate, [a] activated using Batroxobin.

Figure 2.
Example of contraction data, macroscopic appearance and histology for all test conditions. - Erythrocytes: blue (blue-green); - White blood cells: dark purple; - Chitosan: light blue.

Figure 3. x40 magnification Toludone blue stain of all studied clot variants. WB: Whole Blood, BM: Bone Marrow, BMAC: Bone Marrow Aspirate Concentrate, [a] activated using Batroxobin. - Erythrocytes: blue (blue-green); - White blood cells: dark purple; - Chitosan: light blue.

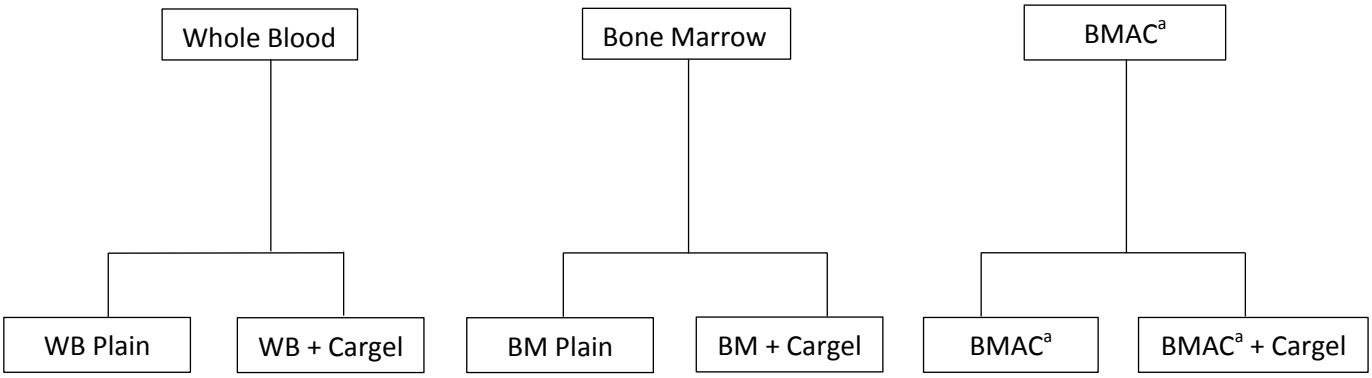
Figure 4.
Live cell confocal fluorescence images (a-e) (z-projections) showing the live (green) and dead (red) cell populations within a single clot from the study. Images a and b were captured at day 1 in two locations in the clot and demonstrate the large number of viable cells spread through lateral cross-section of a clot. Images c, d and e represent clots at day 3, 7 and 10, respectively where good cell viability was maintained. Cell density appears to decrease gradually as the clots gradually break up in culture. The viability plot (f) presents the average cell viability across all samples grouped by timepoint with error bars representing range of viability values.
~~Figure legends~~

~~Figure 1.~~
~~Example of contraction data, macroscopic appearance and histology for all test conditions. - Erythrocytes: blue (blue-green); White blood cells: dark purple; Chitosan: light blue.~~

~~Figure 2. x40 magnification Toludone blue stain of BST-CarGel® - Bone marrow concentrate Batroxibin, showing example of blue patches within the clot. Erythrocytes: blue (blue-green); White blood cells: dark purple; Chitosan: light blue.~~

~~Figure 3.~~

~~Live cell confocal fluorescence images (a-e) (z-projections) showing the live (green) and dead (red) cell populations within a single clot from the study. Images a and b were captured at day 1 in two locations in the clot and demonstrate the large number of viable cells spread through lateral cross-section of a clot. Images c, d and e represent clots at day 3, 7 and 10, respectively where good cell viability was maintained. Cell density appears to decrease gradually as the clots gradually break up in culture. The viability plot (f) presents the average cell viability across all samples grouped by timepoint with error bars representing range of viability values.~~


Whole Blood
Bone Marrow
BMAC[a]
WB Plain
WB + Cargel
BM Plain
BM + Cargel
BMAC[a]
BMAC[a] + Cargel

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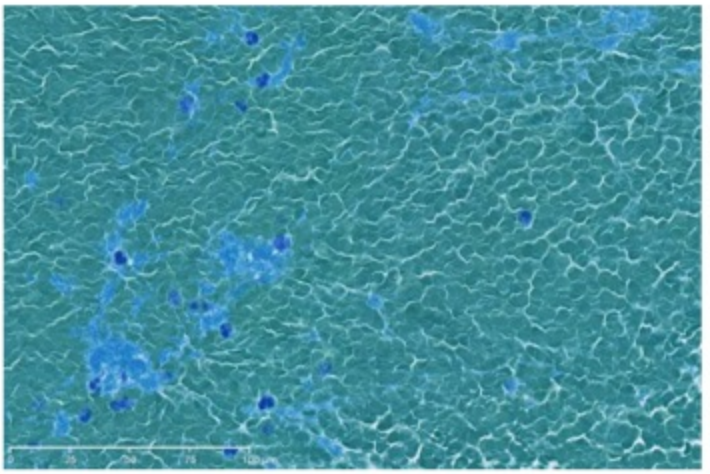
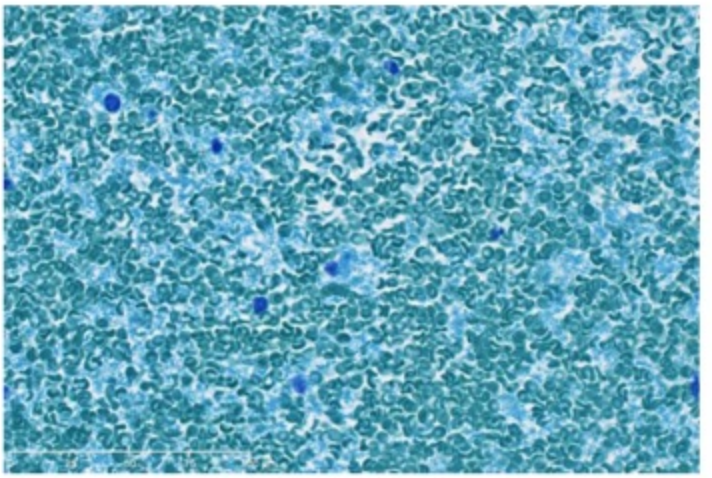
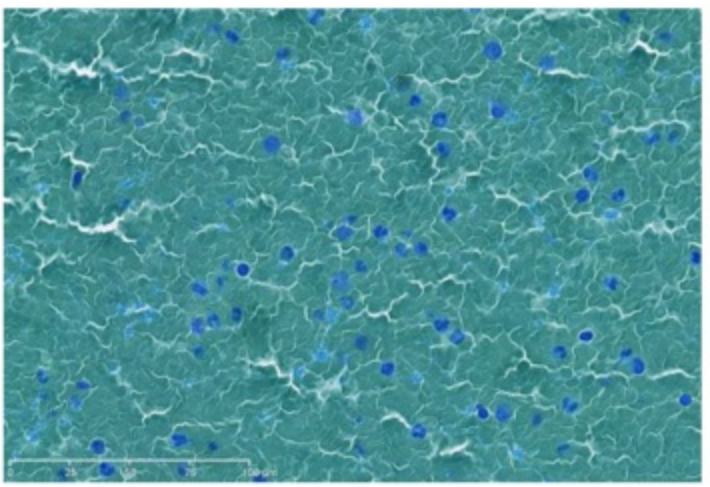


WB



WB + Cargel



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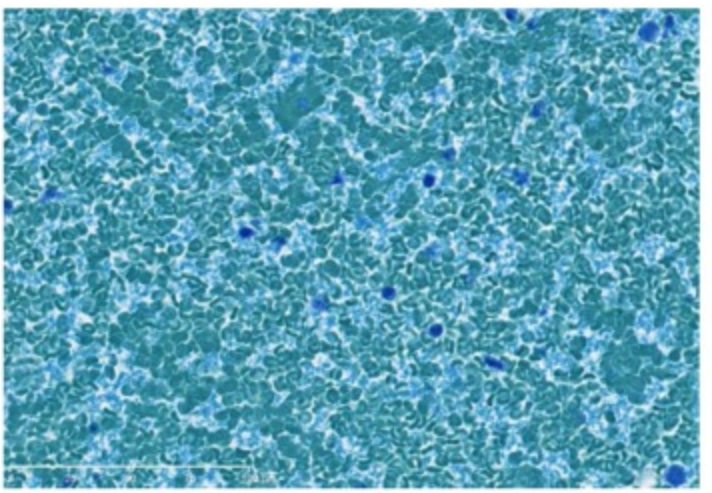
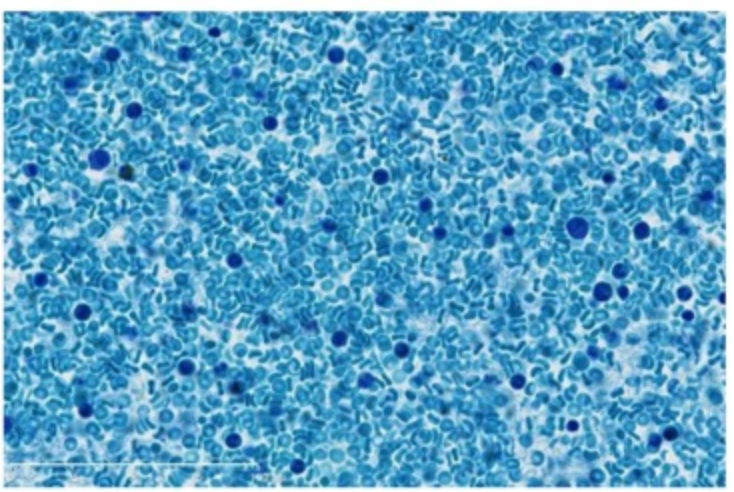
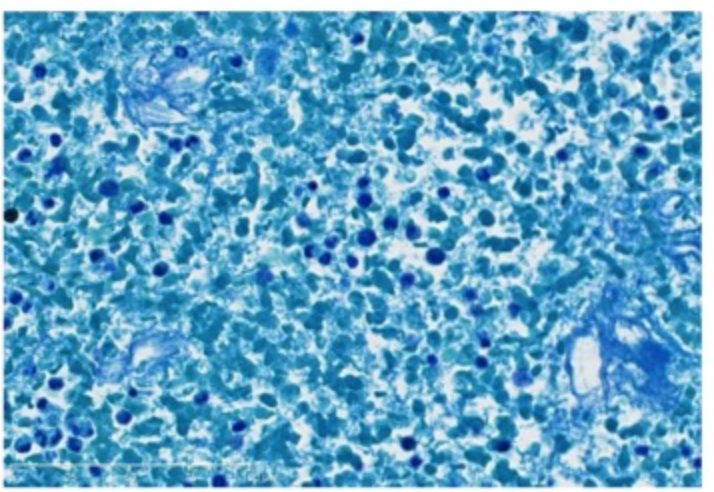


BM + Cargel



BMAC[a]



BMAC[a] + Cargel