COMMONLY USED INTRA-ARTICULAR MEDICATIONS AFFECT BONE MARROW-DERIVED MESENCHYMAL STEM CELL GROWTH AND VIABILITY

A Thesis

by

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ABSTRACT

The objective of this work was to describe bone marrow-derived mesenchymal stem cell (MSC) proliferation and characterization after expansion with the common intra-articular medications polysulfated glycosaminoglycan, hyaluronic acid, or hyaluronic acid with chondroitin sulfates C4 and C6 with N-acetyl-D-glucosamine *in vitro* as an initial screening for combination therapy treatment of degenerative joint conditions in the horse.

MSCs were isolated from bone marrow, then expanded in basal culture media (BCM) or BCM supplemented with a test solution (polysulfated glycosaminoglycan (PSGAG); hyaluronic acid (HA); hyaluronic acid, chondroitin sulfates C4 and C6, and N-acetyl-D-glucosamine (GAGHA)). Viability was assessed with colony forming unit counts (CFU-F), while proliferation assessments included total cell counts, evaluation of growth kinetics with generation tracking intracytoplasmic dye, and culture evaluations of confluency and debris. Characterization by immunophenotyping for surface markers CD29, CD44, CD45, and MHCII, evaluation of differentiation ability into adipocytes, chondrocytes, and osteoblasts, and morphology scoring in culture was performed. Investigation of immunomodulatory activity was assessed by quantification of prostaglandin E_2 (PGE_2) concentration in media supernatants, and senescence was evaluated as a follow-up test in cells expanded with PSGAGs or SF treated media with β-Galactosidase staining (n = 1).

Results of total cell counts, growth kinetics assessment, and culture evaluations for confluency and debris indicated decreased cellular proliferation 72 hours after supplementation with PSGAG, GAGHA, and SF. Viability was decreased in CFU-F cultures supplemented with GAGHA and SF, while colonies failed to form in PSGAG supplemented cultures. Viability was increased in CFU-F cultures supplemented with HA. After 120 hours of expansion with test solutions, there were no significant differences in trilineage differentiation, immunophenotype, or PGE_2 assays. SF cultures stained positively for β-Galactosidase activity and osteogenesis after 10 days of incubation ($n = 1$).

MSCs expanded with PSGAG, GAGHA, and SF had decreased proliferation and viability *in vitro*, while proliferation of MSCs cultured with HA was not different from controls. MSC viability and proliferation is inhibited in the presence of PSGAG *in vitro*. In contrast, viability of MSCs may be improved in the presence of HA, and proliferation is not adversely affected. The results of this study warrant *in vivo* evaluation.

DEDICATION

This work is dedicated to my loving husband, who inspires me daily. Thank you for supporting and helping me to achieve my goals. You are truly my partner in life and irreplaceable encouragement, and this work would not be possible without you.

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NOMENCLATURE

OA	Osteoarthritis
SJ	Synovial Joint
SF	Synovial Fluid
ECM	Extracellular Matrix
HA	Hyaluronic Acid
PSGAG	Polysulfated Glycosaminoglycans
PGE_2	Prostaglandin E_2
NSAID	Non-Steroidal Anti-Inflammatory Drug
DMOAD	Disease-Modifying Osteoarthritis Drug
AAEP	American Association of Equine Practitioners
GAGHA	Polyglycan (hyaluronic acid + chondroitin sulfates C4 & C6 + N-acetyl-D-glucosamine)
MSC	Mesenchymal Stem Cell or Multipotent Stromal Cell
BCM	Basal Culture Media
CTV	CellTrace® Violet
CFU-F	Colony Forming Unit – Fibroblastic
CD29	Integrin β-1
CD90	Thy-1
MHCII	Major Histocompatibility Complex Class II

1. INTRODUCTION

Equine athletes endure substantial stress to joints during training and competition. Stress resulting in acute or chronic inflammation can lead to joint disease or osteoarthritis (OA), which causes approximately 60% of lameness in horses and often removes athletes from competition[1,2]. Joint disease and OA affect synovial joints (SJ) in the appendicular skeleton in joints such as the carpal, metacarpophalangeal, proximal interphalangeal, distal interphalangeal, medial and lateral femorotibial and femoropatellar, and tarsal joints[2,3].

1.1 Joint Anatomy

SJs are connections between bones, comprised of articular cartilage with underlying subchondral bone, surrounded by a joint capsule, lined by synovial membrane, and lubricated by synovial fluid (SF)[4]. Articular cartilage is comprised of hyaline cartilage extracellular matrix (ECM), which absorbs shock and provides smooth surfaces for gliding of bones[4].

Articular cartilage matrix is supported by SF, an ultrafiltrate of the blood, as it is an avascular, multi-layered tissue with decreasing cell density and increasing matrix density towards the articular surface[4]. The dynamic composition of chondral ECM allows its unique function. Collagen, a tripolypeptide arranged in a lattice network, resists pulling forces and anchors cells, while lamina adds integrity to the collagen network of the basement membrane[4]. Fibronectin is a repeated protein with specific

interaction regions such as the RGD loop in the cell binding domain, which binds cells via integrin molecules[5]. Proteoglycans play an essential role in articular cartilage function, with aggregates providing gel-like support and integrity to the ECM[4]. Hyaluronic acid (HA), a high molecular weight glycosaminoglycan ($\geq 1 \times 10^6$ Da) composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, is naturally secreted into joints by mesenchyme to provide lubrication and shock absorption through viscoelasticity, and is a major component of chondrocyte-produced ECM[4,6-10]. Naturally occurring polysulfated glycosaminoglycans (PSGAG) such as keratin sulfates and chondroitin sulfates are held together by a core protein linked to HA to form proteoglycan aggregates in the ECM[4]. Water helps to cushion mechanical loading of the joint, attracted into the cartilage by the strong negative charge of sulfate residues in aggrecan monomers[4]. These elements together allow articular cartilage to withstand the simultaneous forces of loading, shear stress, and compression during locomotion.

1.2 Joint Pathology

Early stage joint disease and OA are often overlooked, as initial damage of aneural articular cartilage may not result in immediate pain and lameness[11]. HA and PSGAG in articular cartilage become depleted, and subchondral bone can be exposed to the articular surface[1]. Hallmarks of OA are osteophyte formation at the joint capsule margin, subchondral bone lysis, and synovial fluid of increased total protein, white blood cell, glycosaminoglycan, and PGE_2 content[12]. The body responds to damage within the

joint cavity with production of inflammatory factors such as prostaglandin E_2 (PGE_2), interleuken-8 (IL-8), IL-1β, tumor necrosis factor-α (TNF-α), and matrix metalloproteinases (MMP) to initiate inflammation[13,14]. Osteoclast recruitment and activity is increased in the presence of PGE_2, resulting in increased bone resorption[13]. MMP-1 and MMP-13 are secreted by synovial cells and chondrocytes, respectively, and contribute to aggrecan and collagen degradation, resulting in lesions of the meniscus and other cartilaginous structures[15]. These degenerative processes trigger additional inflammatory cascades, resulting in an influx of pro-inflammatory cytokines, joint effusion, and increased pain[13]. SF loses viscosity under inflammatory conditions, as high molecular weight HA degrades into smaller fragments, which accumulate and have opposite effects within the joint than the larger molecular form[9,10]. In all, severe injury or degeneration of SJ components triggers an amplifying cascade, resulting in complete loss of joint function if untreated.

Due to the avascular nature and limited cell population of articular cartilage, repair is a slow and rarely complete process[16]. Therefore, degenerative processes driven by chronic inflammation present a greater challenge to healing and regeneration than acute injuries of cartilaginous structures. There are several treatment options to minimize inflammation and pain in both chronic and acute cases, as well as address the disease process.

1.3 Conventional Treatment Options

Though the disease itself is not reversible, treatments decreasing inflammation and pain can lessen the impact of symptoms and hinder disease progression. The least invasive options are external or topical treatments such as extracorporeal shockwave therapy or topical non-steroidal anti-inflammatory drugs (NSAIDs)[2,17]. Both of these options are pain modulating, but offer no disease modification[2]. Systemic treatment with NSAIDs or selective NSAIDs can provide long-term benefits and slow progression of OA in cases of chronic inflammation by inhibiting cyclooxygenase enzymes[18]. Corticosteroids can be injected directly into the joint, and have anti-inflammatory effects by inhibiting phospholipase A_2 function, thereby inhibiting liberation of arachidonic acid from the plasma membrane of cells and inhibiting production of prostaglandins, thromboxanes, and leukotrienes[2]. However, chronic NSAID intra-articular administration demonstrated fibrosis of cartilaginous structures in the rat model, and short-term exposure of equine chondrocytes to corticosteroid resulted in decreased proteoglycan production in another study[19,20]. A human study of intra-articular corticosteroid administration for treatment of OA indicated a risk of increased cartilage loss compared to untreated joints, while an equine study revealed increased aggrecan turnover, cleavage of collagen I and II, and type II collagen synthesis following repeated intra-articular administration of corticosteroid in treated and contralateral control joints[21,22]. These findings indicate local and systemic adverse effects of corticosteroids on articular cartilage. Therefore, intra-articular treatment with these medications should be used with caution in cases with cartilage damage.

Alternatively, potentially disease modifying osteoarthritis drugs (DMOAD) such as Adequan, HA, and Polyglycan have become popular treatments to slow disease progression. In a 2009 survey, Ferris et al. reported Adequan to be the most frequently used DMOAD among American Association of Equine Practitioners (AAEP) member respondents (62.8%)[23]. Among those respondents, Adequan was used as a prophylactic treatment, in chronic cases, and post-surgically, administered intramuscularly (IM) the majority of the time (84.1%)[23]. HA in the form of Legend (10 mg/mL) was used second most frequently (57.4%), followed by Hylartin-V (10 mg/mL), and Hyvisc (11 mg/mL) for various applications[23]. Finally, 18% of respondents reported using Polyglycan (GAGHA) intra-articularly for OA treatment, though it is not labeled for intra-articular injection in the United States, but as a post-surgical intra-articular lavage[23]. Hyaluronan products were most commonly used for combination with corticosteroids (59.4%), followed by the antibiotic amikacin sulfate (56.9%) and Adequan (4.7%)[23].

HA occurs naturally within the joint at a high average molecular weight in the homeostatic state[2,14]. This component of cartilage and synovial fluid can complex with the glycoprotein lubricin to form boundary lubricant complexes which reduce friction during movement, and is present in synovial fluid, providing its viscoelastic property[14]. In the disease state, HA in the joint environment becomes depleted, therefore intra-articular supplementation can be beneficial[2]. Supplementation of high molecular weight HA stimulates endogenous HA production by synoviocytes, provides anti-inflammatory action by inhibiting macrophages and lymphocytes, reduces prostaglandins and catabolic enzymes, improves SF viscosity, and reduces pain[2]. Anti-inflammatory effects of HA

are believed to be CD44 membrane receptor mediated, and occur through suppression of LPS-induced COX-2 production of PGE_2[14]. Downstream effects of CD44 mediated anti-inflammatory action include downregulation of IL-8, TNF-α, IL-1β and therefore the ADAMTS protease enzyme family, resulting in decreased aggrecan degradation[14]. Anti-nociceptive actions after HA administration are also documented[14].

PSGAG supplementation provides chondroprotection and some anti-inflammatory action by inhibiting degradation signaled by inflammatory cytokines and prostaglandins[2]. Chondroprotection, or the protection of tissues from catabolic activity within the inflamed joint, has been demonstrated in several animal models, including the rabbit, dog, and horse, by means of physical, histological, and radiographic assessments[24]. Glade et al. revealed a stimulating effect of PSGAG on chondrocyte cultures, which had increased hyaline ECM production when cultured with PSGAG[25]. Counterintuitively, proliferation of chondrocytes in the presence of PSGAG was inhibited in this study[25]. A study of PSGAG and HA in treatment of an induced OA model revealed that PSGAG was superior to HA in reducing synovial effusion, synovial membrane vascularity, and fibrosis of the subintima[26]. This medication is helpful for slowing disease progression, but does not reverse the disease process.

GAGHA also provides some disease modification according to a study conducted in 2013, which demonstrated modest improvements in lameness, cartilage erosion, and bone proliferation, however, synovial fluid PGE_2 was not reduced[27]. In the same study, horses treated with HA also saw no reduction in PGE_2, but gross articular cartilage fibrillation was reduced. The persistence of PGE_2 suggests that alternate means

besides PGE_2 reduction produced the clinical sign improvements observed with GAGHA and HA treatment[26,27]. This is in contrast to treatment with corticosteroids or IL-1 antagonist applied via gene therapy, which reduced synovial fluid prostaglandin significantly when studied in the same model of OA[28,29]. The 2013 study indicates GAGHA or HA as possible alternatives for cases nonresponsive to corticosteroids or IL-1 antagonists, though reduction in lameness with corticosteroids (56% reduction) or IL-1 antagonists (63% reduction) was greater than that achieved with GAGHA (16% reduction)[27].

1.4 Mesenchymal Stem Cells used in Regenerative Medicine

Regenerative medicine has many applications for both human and animal patients, including treatment of diseases afflicting the musculoskeletal, cardiovascular, and nervous systems[30,31]. The field of regenerative medicine studies the body's many methods of healing to manufacture artificial alternatives for replacing or supplementing the body with cells, tissues, and organs for the treatment of various diseases[32].

The equine model is recognized by the US Food and Drug Administration and the European Medicines Agency as an ideal translational model for musculoskeletal disease in humans[31,33,34]. The horse provides the advantage of a more comparative model than that in mice or rabbits, as joint anatomy, load proportion, and injury and disease state are more similar to that occurring in humans[31,35,36]. Further, cell-based therapies applicable to human joint pathologies are most appropriately demonstrated in the equine model[37].

Intra-articular injection of autologous mesenchymal stem cells (MSCs) is a viable treatment option for joint disease and OA patients[38]. MSCs have low major histocompatibility complex (MHC) expression, therefore have a low propensity to cause an immune response post-injection, and have the capacity to respond to their environment[39,40]. MSCs are capable of changing to a pro-inflammatory or anti-inflammatory phenotype depending on local signals[40]. They secrete a range of soluble factors, influencing endogenous cell proliferation and function, angiogenesis, tissue repair, and inflammation[41]. The exact mechanisms of action and possible extent of MSC therapy are yet to be discovered, and are currently being investigated.

Intra-articular MSC administration is currently used to provide trophic, immunomodulatory and anti-inflammatory benefits to patients with joint disease[42]. Trophic effects are believed to be due to the release of various paracrine trophic factors, growth factors, and chemokines, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), stem cell factor (SCF), stromal cell-derived factor-1 (SDF-1), and others[43]. These factors promote wound healing, tissue repair, and angiogenesis in damaged tissues by signaling endogenous cells such as chondrocytes, synoviocytes, and endothelial cells when injected into the injured or diseased joint[43]. MSCs are similarly capable of modulating immune cells and inflammation[43]. Studies have shown that macrophages can be induced by MSCs to secrete IL-10, or human cytokine synthesis inhibitory factor, and that MSC secretion of soluble factors such as transforming growth factor-β (TGF-β) and PGE_2 can suppress natural killer (NK) cell, T cell, and B cell activity and proliferation[43].

Barrachina et al. demonstrated that MSCs primed by an inflammatory environment containing pro-inflammatory factors TNFα and interferon-γ (IFNγ) respond, at least in part, by upregulating immunoregulatory-related genes such as vascular adhesion molecule 1 (*VCAM-1)*, indoleamine 2,3-dioxygenase *(IDO)*, inducible nitric oxide synthase *(iNOS)*, *IL-6*, and cyclooxygenase-2 (*COX-2)*[42]. Production of soluble factors such as these alters the actions of surrounding cells drives the environment towards a homeostatic state of balanced catabolic and anabolic processes[40]. Berebichez-Fridman et al. report that studies of intra-articular injection of MSCs in pre-clinical osteoarthritis models have demonstrated results including clinical improvement, evidence of chondroprotection, inhibition of osteoarthritis progression, and decreased PGE_2 in synovial fluid[12]. Specifically, a study in a caprine model of posttraumatic osteoarthritis demonstrated reduced articular cartilage degeneration and osteophyte remodeling six weeks after MSC injection[44]. They proposed that injected MSCs induced regeneration of meniscal tissue by endogenous cells via paracrine signaling, as implanted cells were present on the surface of regenerated tissue, but not within[44]. These findings and others demonstrate the prospect of MSCs as a biological therapy to promote tissue regeneration and moderate inflammation in the injured or diseased joint environment through paracrine activity[45,46].

1.5 Mesenchymal Stem Cell Identity

In general, stem cells are defined by the two unique features of self-renewal and potency. Self-renewing stem cells are capable of multiple divisions, which produce identical daughter cells[47]. This type of division allows replication and propagation. Potency refers to the stem cells' capability to remain undifferentiated until receipt of signals from their niche or culture environment[47]. Totipotent stem cells, the result of oocyte fertilization, are capable of differentiating into all embryonic and placental cell types[47]. These cells have the greatest capacity to differentiate and therefore the greatest potency of all stem cell types. Pluripotent stem cells derive from totipotent cells and develop into the three germ layers forming the embryo[47]. Multipotent stem cells have the capacity to differentiate into a close family of cell types, while unipotent stem cells can only produce one type of cell, their own, and are distinguished as stem cells in a specific tissue due to their self-renewing capability through asymmetrical division[47]. Therefore, adult stem cells are either multipotent or unipotent. MSCs have the greatest potency of adult tissue-derived stem cells, first described as osteogenic precursors by the work of Friedenstein et al. in 1966, and first described as having multi-lineage potential by Pittenger et al. in 1999 due to their ability to differentiate into several different cell types of the mesenchymal lineage[48]. MSCs can be found in all tissues of the body, and can be harvested from peripheral blood, synovial fluid, adipose tissue, and bone marrow[49]. After harvest from adult tissues, MSCs can be maintained or manipulated in culture.

Bone marrow houses a heterogeneous population of cells, including MSCs, hematopoietic stem cells (HSCs), and adipocytes, supplying the body with a continuous supply of hematopoietic and mesenchymal lineage precursors[47,50,51]. MSCs secrete fluid factors into the perivascular niche to support HSC maintenance along with adhesive interactions[52,53]. The stem cell niche also provides MSCs with signals and growth factors necessary to prevent differentiation while allowing asymmetrical division, delivering cells to the body as needed without depleting the supply pool[54]. MSCs are isolated in culture from bone marrow harvested from sternal bodies or the ilium in horses[55].

Equine MSCs are characterized in culture by plastic adherence, the presence or absence of specific cell surface markers, and the capability to differentiate into lineages of the mesenchyme, such as osteoblasts, chondrocytes, and adipocytes, *in vitro*[56,57]. They are morphologically spindle-shaped and fibroblast-like, and proliferate in monolayer in culture[57]. The criteria of plastic adherence and capacity for trilineage differentiation are uniform across species, however, the requirements of the immunophenotypic profile of equine MSCs have not been defined, as equine-specific antibodies are not widely available[30,31,57,58].

Immunophenotyping of cell samples ensures the presence of MSCs and the absence of HSCs or any other cell population due to differences in antigen presentation[57]. According to guidelines set forth by the International Society for Cellular Therapy (ISCT), human MSCs express CD105, CD73, and CD90, and lack CD45, CD34, CD14, CD19, and HLA-DR expression[56,58]. No specific panel has been

assembled for equine MSC identification, as commercially available specie-specific antibodies are not widely available[57]. According to others, equine MSCs should express cellular protein markers CD29, CD44, and CD90, and lack expression of CD45 and MHCII[57,59]. Other markers have been added or substituted in other studies to verify isolation and specie-specific identity in culture[31,57]. Integrin β-1, or CD29, is a surface adhesion and recognition molecule involved in multiple processes including the immune response and tissue repair[60]. This surface molecule is common in fibroblast-like cells, and highly expressed in MSCs[60]. CD44 is involved in cell adhesion, migration, interaction, and proliferation, is a receptor for hyaluronic acid (HA), and can promote T-lymphocyte activation[31,61,62]. CD90, also known as Thy-1, is a regulator of cell-to-cell and cell-to-matrix communications during migration and fibrosis and can also promote T-lymphocyte activation[63]. CD45 is a hematopoietic (B-cell) marker, while major histocompatibility complex class II, or MHCII, is found on antigen-presenting cells which initiate immune reactions[64,65]. Low expression of MHCII is thought to provide MSCs with immunoprivilege, a key in their immunomodulatory actions and use for injection[65,66].

Multiple studies exist demonstrating that MSCs cryopreserved in liquid nitrogen show no change in morphology, proliferation, cell surface marker profile, or trilineage differentiation potential[67-69]. The use of cryopreservation facilitates clinical and research applications of MSCs.

2. PROBLEM

2.1 Combination Therapy

Intra-articular medications and regenerative medicine may be combined as an effective treatment approach for OA patients, resulting in enhanced disease modification, allowing the animal to return to training or competition faster. However, combination of MSCs with the intra-articular medications Adequan and Polyglycan has not been studied previously to determine their effects on MSC viability, proliferation, characterization, and immunomodulation.

2.2 Preliminary Data

A pilot study was conducted to evaluate effects PSGAG, HA, and GAGHA on

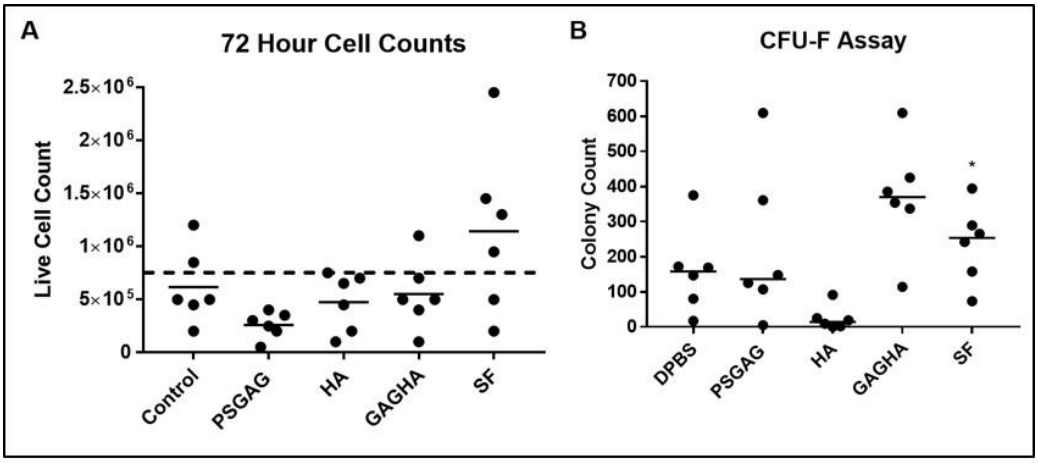

Figure 1 – Preliminary Data 1

MSC proliferation and characterization *in vitro*. It was hypothesized that MSC proliferation and characterization would not change after exposure to these test solutions. Cryopreserved MSCs from 6 horses of various age, breed, and passage were thawed as previously described, stained with CellTrace™ Violet[a] cytoplasmic dye, aliquoted, resuspended in 1mL per test solution (Dulbecco's phosphate buffered saline (DPBS, control), Adequan[b] (PSGAG; 100 mg/mL polysulfated glycosaminoglycan), HA[c] (11 mg/mL hyaluronic acid sodium salt), Polyglycan[d] (GAGHA; 5mg/mL hyaluronic acid sodium salt, 100mg/mL sodium chondroitin sulfate, 100mg/mL N-acetyl-D-glucosamine), or pooled SF) for 5 minutes, then plated in the resuspension medias to tissue culture flasks (10,000 cells/cm^2) and 10cm culture dishes (1000

[a] ThermoFisher Scientific, Rochester, NY, USA
[b] Lutipold Pharmaceuticals, Shirley, NY, USA
[c] Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, USA
[d] ArthroDynamic Technologies, Lexington, KY, USA

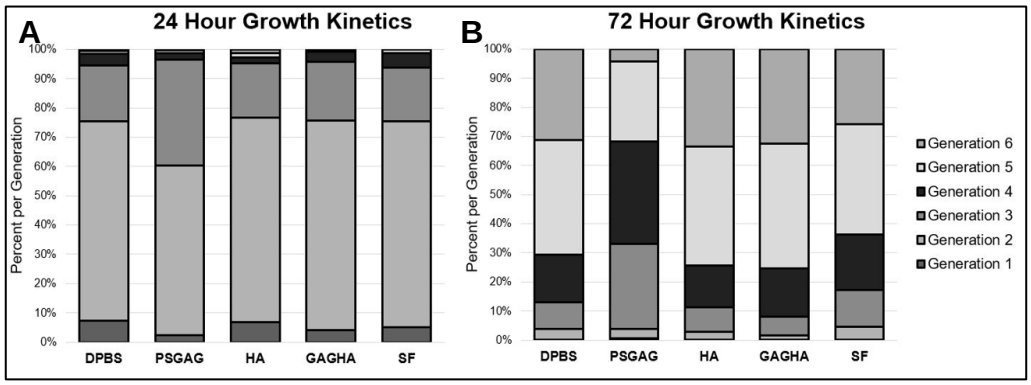

Figure 2 – Preliminary Data 2

cells/dish) with basal culture media (BCM) for the colony forming unit-fibroblastic (CFU-F) assay[69]. Half of the media was replaced in each flask with BCM after 48 hours of incubation. Cell counts and flow cytometry were performed at 24 and 72 hours to assess growth kinetics, and 10cm plates were incubated for 10 days, then stained with Crystal Violet to assess viability. Cell counts, growth kinetics, and CFU-F data were analyzed with commercially available software[e] with the Shapiro-Wilk distribution test and repeated measures one-way ANOVA with Dunnett's multiple comparisons test. P-values less than 0.05 were considered significant. Though no statistically significant differences were found between test solution groups and the control within cell count and growth kinetics data, MSCs expanded with PSGAG appeared to have decreased cell counts and a greater proportion of cells in earlier generations compared to the control and other treatments at 72 hours (Figure 1, Figure 2). The CFU-F assay indicated significantly increased viability of MSCs exposed to pooled SF, and decreased viability

[e] GraphPad Software, Inc., La Jolla, CA, USA

when cultured with PSGAGS and HA (Figure 1). The extremely viscous nature of HA could be responsible for the decreased cell counts and CFUs, as much of the cell suspension was retained in the pipette tip during seeding of flasks and plates. Overall, preliminary data showed the proliferation of MSCs combined with PSGAG was hindered. Our findings suggested that further investigation was warranted with adjusting the experimental techniques to more closely mimic clinical application. In addition, adding assays to evaluate effects on MSC characterization and immunomodulatory function would provide greater knowledge of the effects of these medications on MSCs.

2.3 Objectives

The objective of this study was to determine the effects of the commonly used intra-articular drugs PSGAG, GAGHA, high molecular weight HA, and SF on bone marrow-derived MSC viability, growth, cell surface marker expression, differentiation ability, and PGE_2 secretion *in vitro*. It was hypothesized that MSCs expanded with PSGAG would have decreased viability and proliferation due to the results of the pilot study, but that characterization would be unchanged for all test solutions.

3. METHODS

3.1 Inclusion Data

MSCs were isolated and expanded as previously described[69]. Donor horse age ranged from 4 – 8 years (median, 7). There were 5 geldings and 1 mare. Passage 2 – 4 MSCs were cryopreserved for 2 – 4 years prior to use. SF was collected post-euthanasia from horses euthanized for reasons other than joint disease. Immediately after collection, SF was spun (300 x g, 5 minutes) to remove cells and other debris. SF was frozen for 0 – 39 days prior to use. No animals were euthanized specifically for the purpose of this study, and animal use procedures were approved by the Texas A&M University Animal Care and Use Committee (IACUC #20150038).

3.2 Test Solutions

Test solutions (Adequan[1] (PSGAG; 100mg/mL polysulfated glycosaminoglycan), hyaluronic acid[f] (HA; 11mg/mL hyaluronic acid sodium salt from streptococcus equi, 1.5-1.8 x 10^6 Da), Polyglycan[3] (GAGHA; 5mg/mL hyaluronic acid sodium salt, 100mg/mL sodium chondroitin sulfate, 100mg/mL N-acetyl-D-glucosamine), or SF) were combined with BCM and then sterile filtered to create a 1 part test solution to 11 parts BCM volumetric ratio for each test solution (Table 1). The original ratio chosen for the project was 5 parts BCM to 1 part test solution to closely mimic the average ratio of joint volume to a dose of HA after intra-articular injection

[f] Sigma-Aldrich, St. Louis, MO, USA

(Table 2). The ratio was adjusted to 11:1 for all test solutions for improved practicality, which still closely mimicked the ratio of joint volume to a dose of Adequan (10:1; Table 2). The 11 mg/mL concentration for HA test solution was chosen to mimic the concentration of the commercially available HA product, Hyvisc[g]. After the first passage post-thaw, MSCs were maintained in BCM with test solution for each of the assays.

Test Solution		Concentration	Concentration in Media
Adequan	Polysulfated glycosaminoglycans	100 mg/mL	8.0 mg/mL
Hyaluronic acid, 1.5 – 1.8 x 10^6 Da		11 mg/mL	0.9 mg/mL
Polyglycan	Hyaluronic acid	5.0 mg/mL	0.4 mg/mL
	Sodium chondroitin sulfate	100 mg/mL	8.0 mg/mL
	N-acetyl-D-glucosamine	100 mg/mL	8.0 mg/mL

Table 1 – Test Solution Concentrations

[g] Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA

Common Name	Joint	Volume[1]	Hyvisc[3]	Adequan[4]	Polyglycan[5]	Approximate Ratio of Joint Injection Volume to Medication		
						Hyvisc	Adequan	Polyglycan
Fetlock	Metacarpophalangeal	12 mL	2 mL (22 mg)		Post-surgical lavage 10 mL: 50 mg HA 1000 mg glucosamine 1000 mg chondroitin sulfate	12:2		
Pastern	Proximal Interphalangeal	6 mL[2]				6:2		
Knee (Carpus)	Radiocarpal Intercarpal	10 mL	2 mL (22 mg)	1 mL (250 mg)		10:2 22 mg	10:1 250 mg	
Coffin	Distal Interphalangeal	6 mL						
Stifle	Femorotibial Femoropatellar	> 20 mL	4 mL (44mg)			20:4		
Hock (Tarsus)	Tibialtarsal Intertarsal	20 mL	4 mL (44 mg)			20:4		
Average volumetric ratio of injection volume to drug dose volume						**4.8:1**	**10:1**	

[1] Moyer W, Schumacher J, Schumacher J. Equine joint injection and regional anesthesia. Academic Veterinary Solutions; 2011.
[2] Schumacher J, Wilhite R. How to make rational choices for intra-articular injections. How to inject the synovial cavities of the digit. 2012; 58:430-438.
[3] Hyvisc product insert.
[4] Adequan product insert. Adequan is indicated for use only in the carpal joint.
[5] Polyglycan product insert.

Table 2 – Approximate joint volumes and intra-articular drug doses. Joint volumes are in reference to recommended volumes of intra-articular anesthetics.

3.3 Growth Kinetics Assay

MSCs were stained with CellTrace™ Violet Cell Proliferation Kit[h] (CTV) according to the manufacturer's instructions. CTV-stained cells were plated to tissue culture flasks (5700 cells/cm^2) with BCM or BCM supplemented with test solution and incubated for 24 and 72 hours, counted, then cryopreserved. MSCs were thawed as previously described and resuspended in 500µL DPBS[69]. Five microliters of 2% PI was added to each sample for viability assessment during flow cytometric analysis. Only live cells were analyzed. Samples were analyzed using a MoFlo Astrios high-speed cell sorter and Summit acquisition software[i]. The flow cytometer used a 405nm laser with a 488/55 bandpass filter. The CellTrace™ Violet label excites at 405nm and emits at 455nm. The data were modeled using ModFit LT software[j], and reported as sample percentages in each generation.

3.4 Colony Forming Unit Assay

MSCs were seeded to one 10cm culture dish (1000 cells per dish) per test solution and incubated. Following 10 days of incubation, the dishes were stained with Crystal Violet, and circular colonies visible to the naked eye were counted.

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[h] ThermoFisher Scientific, Rochester, NY, USA
[i] Beckman Coulter, Indianapolis, IN, USA
[j] Verity Software House, Topsham, ME, USA

3.5 Culture Evaluations

One flask per test solution was imaged at 24, 72, and 120 hours by phase contrast microscopy at 40x total magnification. When all test solution cultures were imaged, the images were randomized, and two evaluators with MSC culture experience graded the images for confluency, morphology, and debris in a blinded manner (Table 3). Due to software difficulty, cultures from 5 horses were assessed for 24 hour culture evaluations, while cultures from 6 horses were assessed for 72 and 120 hour evaluations.

Score	Morphology	Debris	Confluency
1	**Excellent**	None	76 – 100%
	Slender, spindle-shaped	*< 5 particles per 40x view*	
2	Good	Mild	51 – 75%
	Spindle-shaped with cytoplasmic projections	*6 - 25 particles per 40x view*	
3	Fair	Moderate	26 – 50%
	Slightly rounded with slightly distended cytoplasm and vacuoles	*26 - 50 particles per 40x view*	
4	Poor	Severe	0 – 25%
	Rounded with very distended cytoplasm, projections, and vacuoles	*≥ 51 particles per 40x view*	

Table 3 – Culture Evaluation Parameters

3.6 Trilineage Differentiation Assay

MSCs expanded with test solutions for 120 hours were subjected to trilineage differentiation to assess potency. For adipogenic differentiation, cells grown in each test solution were seeded to 12-well plates (1000 cells/cm^2) in triplicate wells with BCM. After 24 hours, the BCM in each well was replaced with adipogenic induction media (Dulbecco's modified Eagle's medium F12 with L-glutamine and sodium pyruvate[k], 3% FBS[l], 10,000 units/mL penicillin, 10mg/mL streptomycin, 25 µg/mL amphotericin B[k], 1 ng/mL bFGF[m], 5% rabbit serum[n], 0.033µM/mL biotin[o], 0.017µM/mL calcium pantothenate[o], 0.001µM/mL insulin[o], 1nM/mL dexamethasone[o], 0.1mg/mL isobutylmethylxanthine[o], and 0.00178mg/mL rosiglithizone[o])[70]. After 3 days, media was aspirated from each triplicate well and adipogenic maintenance media (adipogenic induction media formulation, less isobutylmethylxanthine and rosiglithizone) was added. After 3 days, the plates were fixed with 4% paraformaldehyde, stained with Oil Red O[o], and imaged immediately by phase contrast microscopy using a 3-hole template. Contamination caused results to be excluded in the PSGAG group for one of the six horses.

For chondrogenic differentiation, cells grown in each test solution were divided into 3 aliquots of 500,000 cells, then centrifuged (300 x g, 4°C, 10 minutes, acc7/dec5) to form pellets. Supernatants were carefully removed from each tube, and 1mL of

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[k] Mediatech, Manassas, VA, USA
[l] HyClone, Inc., Logan, UT, USA
[m] Corning, Bedford, MA, USA
[n] Life Technologies, Foster City, CA, USA
[o] Sigma-Aldrich, St. Louis, MO, USA

chondrogenic media was added (Dulbecco's modified Eagle's medium 4.5g/dL glucose with L-glutamine and sodium pyruvate[k], 1% FBS[l], 10,000 units/mL penicillin, 10mg/mL streptomycin, 25 μg/mL amphotericin B[k], 0.01μg/mL transforming growth factor β-3[o], 0.1nM/mL dexamethasone[o], 0.05mg/mL L-ascorbic Acid[o], 0.04mg/mL proline[o], and 1% ITS Premix[p])[69]. The pellets were maintained in culture for 21 days with incubation (37°C, humidified air with 5% CO_2) and media replacement every 2 – 3 days. At the end of 21 days, media was removed pellets were fixed with 4% PFA (room temperature, 10 minutes). The pellets were preserved in 70% ethanol until embedding, sectioning, and mounting to slides. Pellet sections were stained with Toluidine Blue[o], imaged by light microscopy at 100x total magnification, and evaluated for cell morphology, distance, and ECM staining intensity.

For osteogenic differentiation, cells grown in each test solution were plated to 12-well plates (1000 cells/cm^2) in triplicate wells. After 24 hours, BCM in each triplicate well was replaced with osteogenic media (Dulbecco's modified Eagle's medium Ham's F12 with L-glutamine and sodium pyruvate[k], 10% FBS[l], 10,000 units/mL penicillin, 10mg/mL streptomycin, 25 μg/mL amphotericin B[k],1 ng/mL bFGF[m], 0.01μM/mL β-Glycerophosphate[o], 0.02 nM/mL dexamethasone[o], and 0.05mg/mL L-ascorbic acid[o]). The plates were maintained in culture for 21 days with incubation and media replacement every 2-3 days. The plates were fixed with 70% ethanol, allowed to dry completely, and stained with 2% Alizarin Red[o]. The plates were allowed to dry completely, then assessed and imaged by phase contrast microscopy.

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[p] Discovery Labware, Inc., Bedford, MA, USA

3.7 Immunophenotyping Assay

MSCs expanded in treated media for 120 hours were analyzed for expression of surface markers CD29, CD44, CD45, and MHCII by flow cytometry as previously described[69]. Briefly, MSCs were thawed and stained using antibody dilutions of 1:100 for CD29[q], CD44[r], and MHCII[n], and 1:33 for CD45[s] with volumes adjusted for cell quantities. MSCs were combined with staining buffer and primary antibodies (CD29, CD44, MHCII) in Eppendorf tubes, incubated (4°C, 45 minutes), pelleted by centrifugation (4 x *g*, 4°C, 5 minutes) and washed twice before resuspension in 500µL DPBS. MSCs were combined with staining buffer and CD45 antibody dilution in Eppendorf tubes and incubated (on ice, in the dark, 15 minutes), then centrifuged (2.0 RPM, 4°C, 3 minutes) and washed twice. Secondary antibody of 1:100 dilution was added to cells, which were then incubated (on ice, in the dark, 15 minutes) and washed once before resuspension in 500µL DPBS.

Five microliters of 7-amino-actinomycin D[t] (7-AAD) was added to each sample for viability assessment during flow cytometric analysis. Only live cells were analyzed. Samples were analyzed using a FACSCalibur flow cytometer and CellQuest Pro acquisition software[u]. The flow cytometer used a 480nm laser with a 530/30 bandpass filter for FITC, 585/45 bandpass filter for PE, and 630 longpass filter for 7-AAD. The

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[q] Beckman Coulter, Indianapolis, IN, USA
[r] Bio-Rad Laboratories, Redmond, WA, USA
[s] Monoclonal Antibody Center, Washington State University, Pullman, WA, USA
[t] BioLegend, San Diego, CA, USA
[u] Becton, Dickinson, and Company, Franklin Lakes, NJ, USA

flow cytometry data was analyzed using FlowJo analysis software[v]. Surface markers with greater than 80% detection were considered positive, while markers with less than 5% detection were considered negative.

3.8 Prostaglandin E_2 Assay

Culture media supernatants of 4 horses were assayed for prostaglandin E_2 (PGE_2) content using a competitive enzyme-linked immunosorbent assay (ELISA)[w]. Absorbance was read at 450nm using a Gen5™ microplate reader and analysis software[x].

3.9 Senescence Assay

MSCs from 1 horse were plated to four 6-well plates (7000 cells/cm^2) in BCM (negative control), high-glucose BCM (24mM glucose, positive control), PSGAG test solution, or SF test solution, and incubated for either 24, 72, 120, or 240 hours. At each time point, plates were stained using a β-Galactosidase Staining Kit[y] according to the manufacturer's instructions, visualized, and imaged by phase contrast and light microscopy.

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[v] Tree Star, Inc., Ashland, OR, USA
[w] R&D Systems, Inc., Minneapolis, MN, USA
[x] BioTek Instruments, Inc., Winooski, VT, USA
[y] BioVision, Inc., Milpitas, CA, USA

3.10 Statistical Analysis

GraphPad PRISM[4] statistical software was used to analyze data. Distributions were evaluated by the Shapiro-Wilk distribution test. Cell count and CFU-F data were evaluated by matched repeated measures one-way analysis of variance (RM one-way ANOVA) with Dunnett's multiple comparisons follow-up test. Culture evaluation data were evaluated for normality with the Shapiro-Wilk distribution test, and either RM one-way ANOVA with Dunnett's multiple comparisons follow-up test or a Friedman test with Dunn's multiple comparisons follow-up test were applied, depending on the nature of the distributions. A p-value of less than or equal to 0.05 was considered significant.

Assay	Plating Density	Length of Expansion
Growth Kinetics	5700 cells/cm^2	24, 72 hours
Cell Counts	5700 cells/cm^2	24, 72 hours
Trilineage Differentiation	10,000 cells/cm^2	120 hours
Immunophenotyping	10,000 cells/cm^2	120 hours
PGE_2	10,000 cells/cm^2	120 hours
Colony Forming Units (CFU-F)	18 cells/cm^2	240 hours

Table 4 – Assay plating densities and lengths of expansion.

4. RESULTS

4.1 Growth Kinetics Assay

There were no significant differences in percentage of cells in each generation after 24 hours of expansion between samples grown in each treated media and the control. After 72 hours of expansion, PSGAG cultures had a significantly greater percentage of cells in generation 2 (median, 43%) and a significantly lower percentage of cells in generation 4 (1.0%) compared to the control (generation 2, 4.0%, generation 4, 41%) (Figure 3). GAGHA cultures had a significantly greater percentage of cells in generation 3 (32%) compared to the control (15%). SF cultures had a significantly greater percentage of cells in generations 1 (9.0%) and 2 (40%) compared to controls (generation 1, 1.0%, generation 2, 4.0%). HA cultures did not have significant differences from the control in any generation.

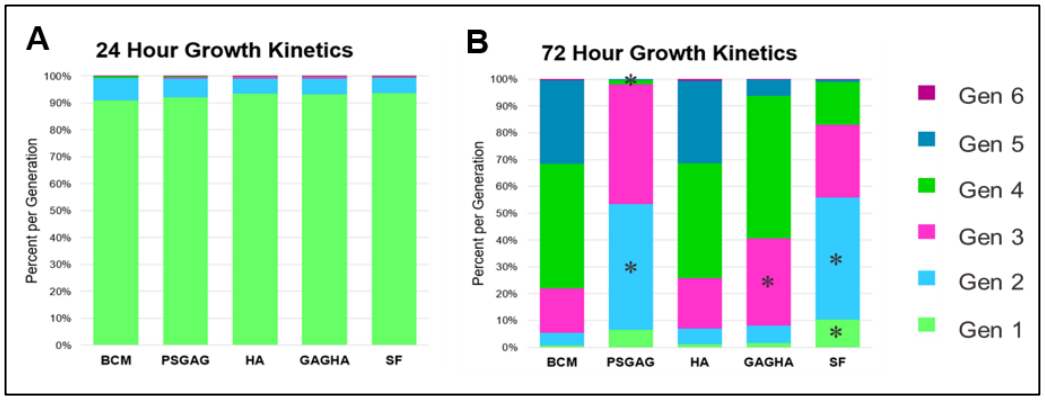

Figure 3 – Growth Kinetics Data.

4.2 Cell Counts

One of the six horses was an outlier and excluded from cell count analysis, though this did not change the results of the statistical tests. There were no significant differences between test solutions and the control at 24 hours (Figure 4). At 72 hours, PSGAG (range, 420,000 – 650,000, mean, 435,000), GAGHA (380,000 – 1,050,000, 556,667), and SF (300,000 – 1,200,000, 508,333) cultures were significantly decreased from the control (800,000 – 2,000,000, 1,268,333) (Figure 4). HA (680,000 – 1,700,000, 1,056,667) cultures were not significantly different from the control at 72 hours.

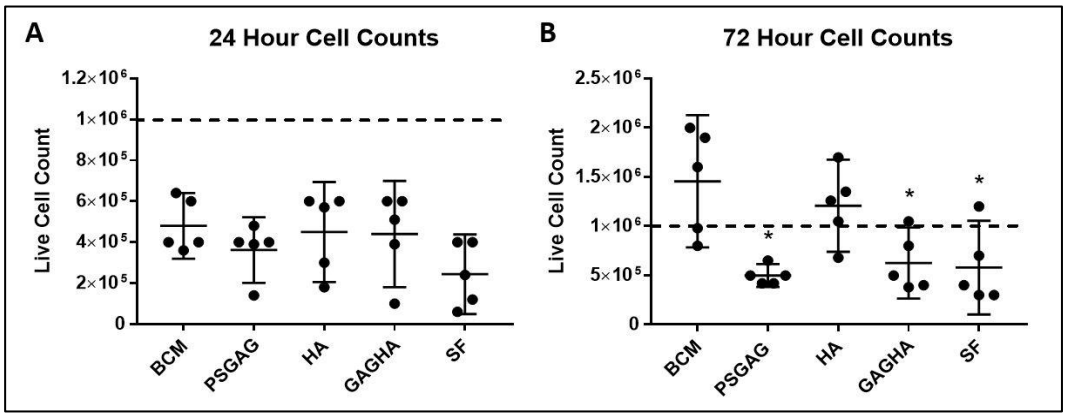

Figure 4 – Cell Count Data (mean, 95% confidence interval).

4.3 Colony Forming Unit Assay

GAGHA (range, 158 - 377, mean 265) and SF (64 – 429, 190) colony counts were significantly decreased, and HA (251 – 652, 414) colony counts were significantly increased from the control (253 – 533, 363) (Figure 5). No colonies were observed on PSGAG test solution plates, though single cells with flattened and circular morphology were visualized and took up Crystal Violet stain (data not shown; Figure 5B). Small colonies observed in SF cultures had unique morphology. As opposed to the circular, evenly distributed colonies observed in control plates (Figure 5C), colonies growing in SF supplemented cultures were tightly packed with defined edges (Figure 5D).

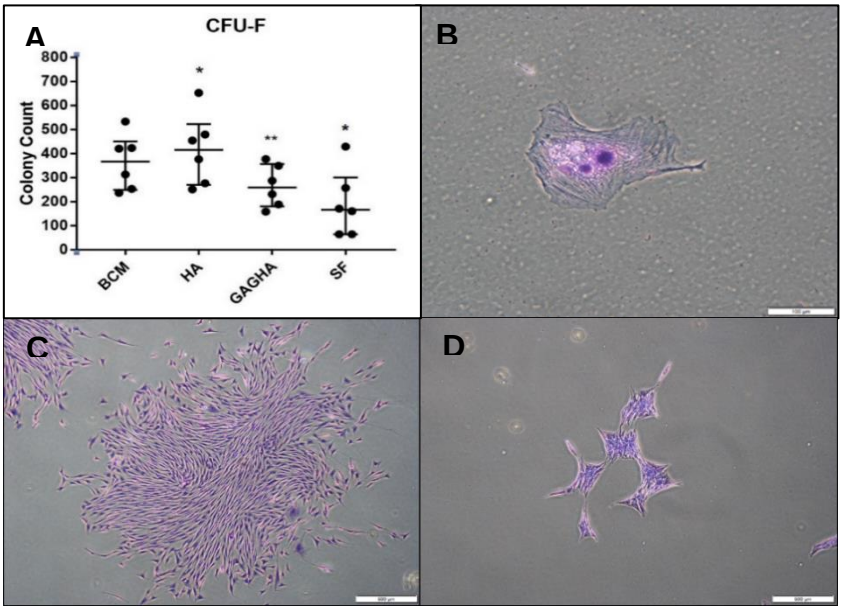

Figure 5 - Colony Forming Units Data. (A) Colony Forming Units –Fibroblastic (mean, 95% confidence interval), (B) representative MSC grown in PSGAG test solution for 10 days, then stained with Crystal Violet, 200x total magnification, (C) colony from BMC group, 40x (D) colony from SF group, 40x).

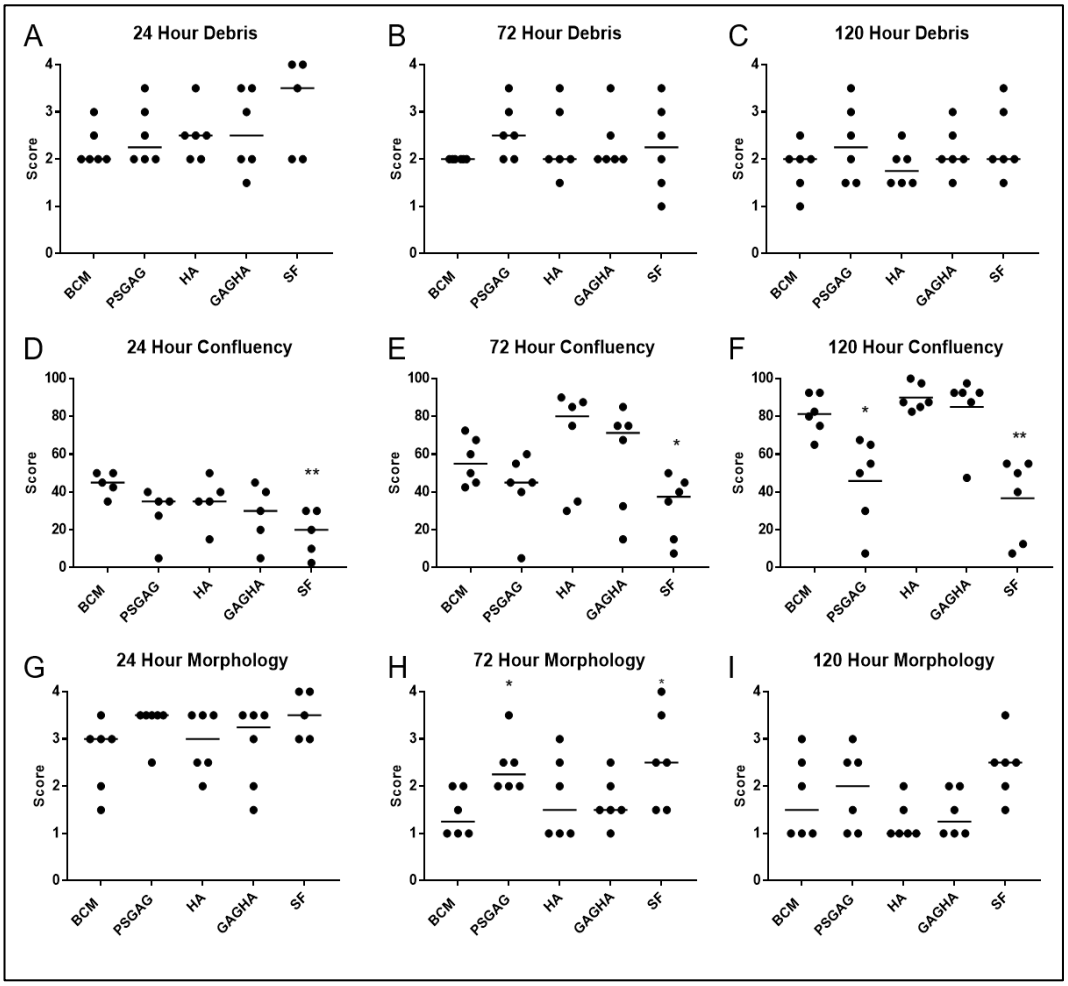

Figure 6 – Culture Evaluation Data (median; 24 hour SF data, n =

4.4 Culture Evaluations

No significant differences were found among test solution groups at 24, 72, or 120 hours for debris in culture. SF cultures were significantly less confluent than controls at 72 and 120 hours, while PSGAG cultures were significantly less confluent than controls at 120 hours (Figure 6). Morphology of these cultures also received worse scores than controls at 72 hours. All other treatments were not significantly different

from controls at any time point for any parameter, however, HA and GAGHA cultures appeared to have greater confluency than controls at 72 and 120 hours.

Test Solution	24 Hours	72 Hours	120 Hours
Debris			
BCM	2.0	2.0	2.0
PSGAG	2.3	2.5	2.3
HA	2.5	2.0	1.8
GAGHA	2.5	2.0	2.0
SF	3.5	2.5	2.0
Confluency			
BCM	44%	55%	81%
PSGAG	31%	45%	53%
HA	35%	80%	88%
GAGHA	25%	71%	93%
SF	20%	38%	45%
Morphology			
BCM	3.0	1.3	1.5
PSGAG	3.5	2.3	2.0
HA	3.0	1.5	1.0
GAGHA	3.3	1.5	1.3
SF	3.5	2.5	2.5

Table 5 – Culture evaluation scores (median; for 24 hour SF scores, n = 5). See Table 3 for parameter descriptions, page 21.

4.5 Trilineage Differentiation Assay

MSCs from all test solution groups successfully underwent trilineage differentiation into adipocytes, chondrocytes, and osteoblasts (Table 6, Figure 7). Although not statistically significant, MSCs expanded in PSGAG supplemented BCM appeared to accumulate less ECM in chondrogenic differentiation as indicated by the distance between cells parameter (Figure 7C).

Assay	BCM	PSGAG	HA	GAGHA	SF
Adipogenesis	+	+	+	+	+
Chondrogenesis	+	+	+	+	+
Osteogenesis	+	+	+	+	+

Table 6 – Trilineage Differentiation Results

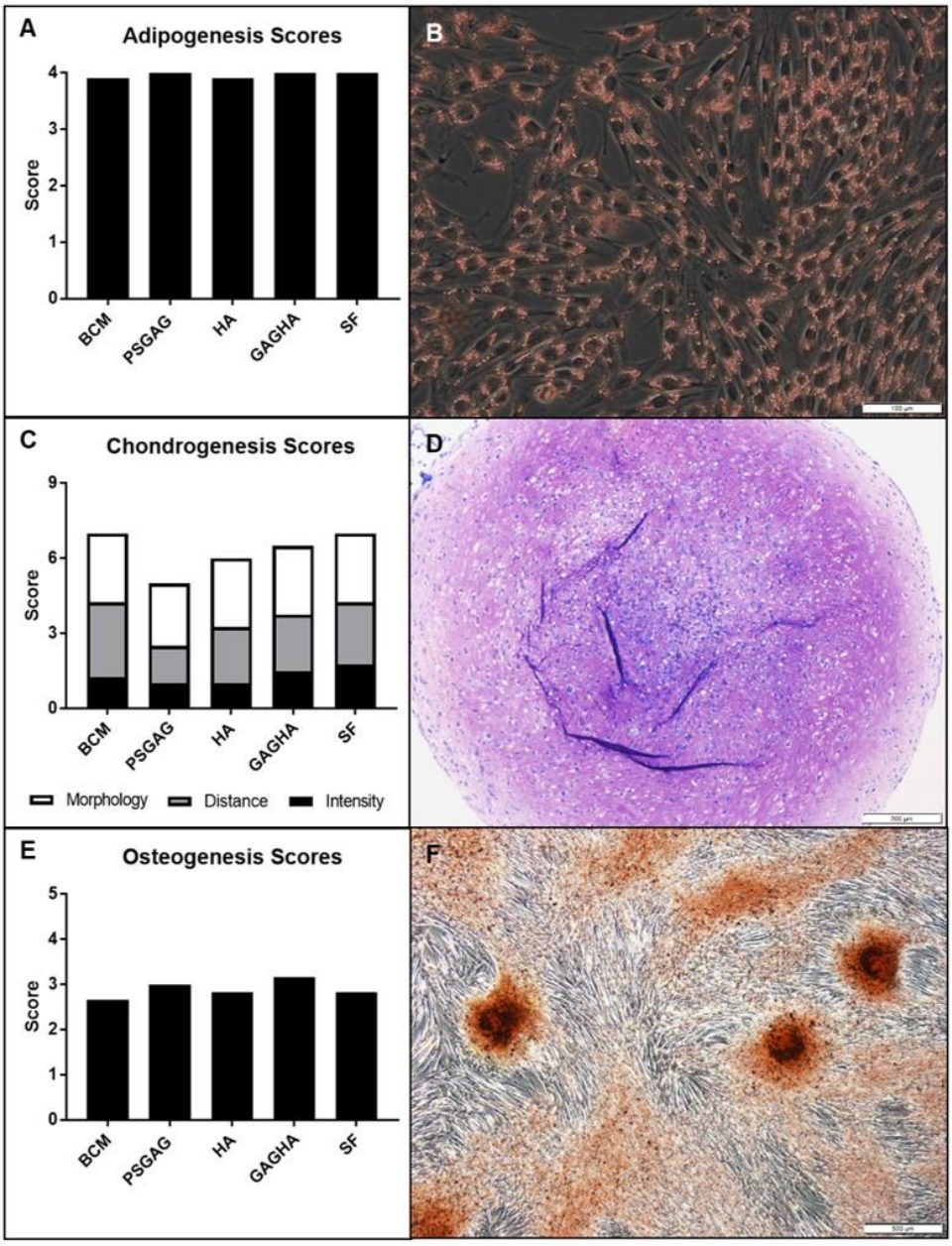

Figure 7 – Trilineage Differentiation Assay Results. (A) Adipogenesis scores, which evaluated the percentage of cells in each view containing stained vacuoles. (B) A high-scoring microscopic view of adipogenic cells at 200x total magnification (Oil Red O). (C) Combined chondrogenesis scores consisting of individual scores for staining intensity, distance between cells, and cellular morphology (medians). (D) A high-scoring chondrogenic tissue section at 100x total magnification (Toluidine Blue). (E) Osteogenesis scores, which evaluated the intensity of stain taken up by the tissue and the presence or absence of nodes. (F) A high-scoring microscopic view of osteogenic cells at 40x total magnification (Alizarin Red S).

4.6 Immunophenotyping Assay

MSCs maintained in culture with treated media for 120 hours showed expected surface marker phenotype of $CD29^{+}$, $CD45^{-}$, and $MHCII^{-}$. Though not significantly different from controls, expression of CD44 appeared to be decreased across test solution groups, with the lowest expression in the PSGAG test solution group (Table 7). Expression of less than 5% was considered negative, while expression of greater than 80% was considered positive.

Marker	BCM	PSGAG	HA	GAGHA	SF
MHCII	-	-	-	-	-
CD29	+	+	+	+	+
CD44	41.1	8.09	24.0	27.3	28.3
CD45	-	-	-	-	-

Table 7 – Immunophenotyping results of cells expanded in treated media for 120 hours, reported in percentages. A positive (+) distinction required greater than 80% expression, while a negative (-) distinction required less than 5.0% expression for all horses (n = 6). CD44 expression was heterogeneous (median).

4.7 Prostaglandin E_2 Assay

Test solution means were not significantly different from the control, however, the range of concentrations for GAGHA culture supernatants (1014.18 – 5779.82 pg/mL) appeared greater than that of the control (0.00 – 1495.44 pg/mL), while the ranges of PSGAG culture supernatants (0.00 – 254.81 pg/mL) and SF culture supernatants (0.00 – 314.14 pg/mL) appeared narrow compared to the control (Figure 8). HA culture supernatants had a moderate range (0.00 – 2878.05 pg/mL). PGE_2 concentrations appeared to be horse-dependent.

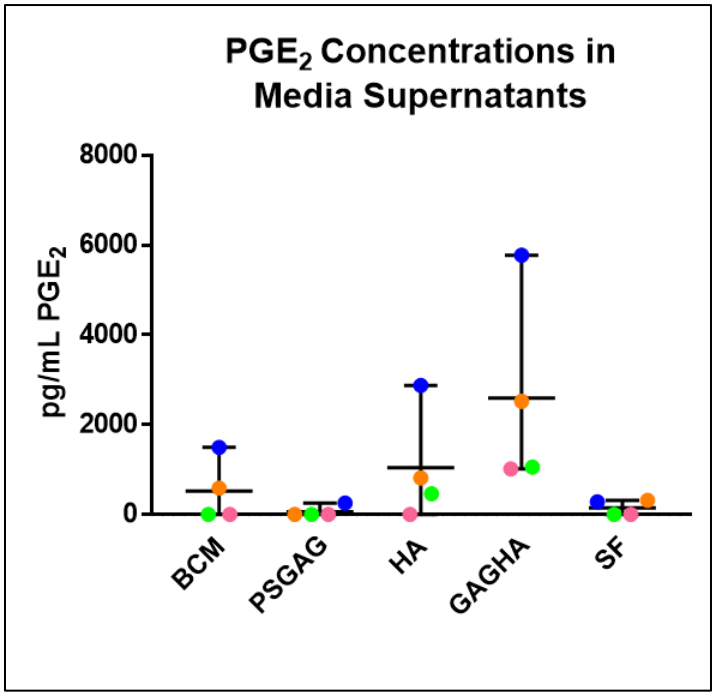

Figure 8 – PGE_2 Concentrations in Media Supernatants (mean, range; Horse 1, blue; Horse 2, pink; Horse 3, green; Horse 4, orange).

4.8 Senescence Assay

Senescence was not detected in control and test solution wells at 24, 72, and 120 hours (n = 1). After 240 hours, positive senescence staining was observed in wells containing MSCs incubated with positive and negative control media and BCM supplemented with SF test solution. Osteogenic differentiation was also observed in SF wells, confirmed by Alizarin Red S staining (Figure 7). PSGAG treated MSCs were negative for β-Galactosidase staining.

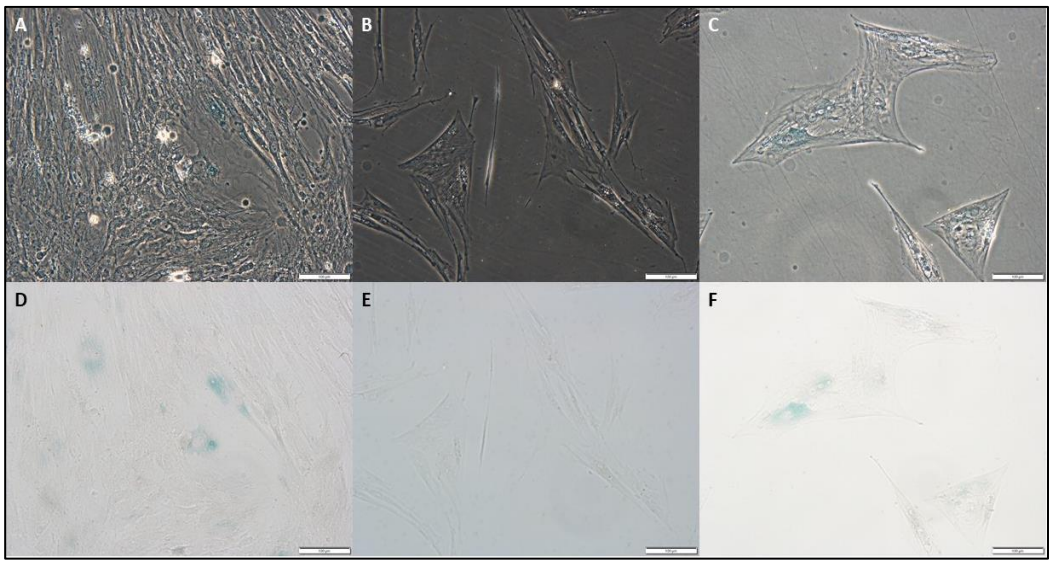

Figure 9 – β-Galactosidase Staining (phase contrast (A-C) and light (D-F) microscopy of MSCs incubated for 240 hours in positive control media (A, D), PSGAG test solution (B, E), and SF test solution (C, F)).

5. DISCUSSION AND CONCLUSIONS

Coupling regenerative medicine with common intra-articular DMOADs may be a valuable therapy for equine joint disease, however, effects of the common DMOADs PSGAG and GAGHA on MSCs were previously unknown. PSGAG, GAGHA, and allogenic SF had negative effects on MSC viability, proliferation, and possibly function. The current study demonstrated no detrimental effects of HA on MSC proliferation, and possibly beneficial effects of HA on MSC viability. These findings indicate that the exposure of MSCs to PSGAG or GAGHA is detrimental to MSCs and that endogenous SF may hinder MSC survival or induce differentiation, while HA had no adverse effects on MSC viability or proliferation.

Our findings are in agreement with those of Bohannon et al. regarding the effects of HA on MSC viability and proliferation, in that HA had no adverse effects on MSC proliferation *in vitro*[71]. The growth kinetics assay indicated no difference in the lag and log phases of growth between HA and control cultures[72]. Confluency scores of HA cultures suggest a possible positive effect of HA on MSC proliferation. Additionally, results of the CFU-F experiment indicate a beneficial effect on MSC viability when plated in low densities. Characterization of MSCs was not altered after 120 hours of expansion to HA, with the exception of CD44 expression, which was decreased in all test solution groups. Our findings of similar PGE_2 production between HA and control cultures are also in agreement with those of the Bohannon study[71]. This suggests that the modulatory functions of MSCs are not hindered after expansion with HA.

Additional investigation of modulator production by MSCs after expansion with HA is warranted.

In contrast, PSGAG cultures performed poorly in all assays. The most conspicuous effects were the change in morphology from spindle-shaped to round and flattened, the delayed recovery after plating demonstrated by confluency scores at 120 hours, and the complete lack of colonies in the CFU-F assay. These observations may be attributed to cell death rather than differentiation or senescence, as the cells lacked morphology and vacuoles indicating adipogenic differentiation or the deposition of ECM characteristic of chondrogenic or osteogenic differentiation, and were negative for senescence after 10 days of culture (n = 1). Decreased proliferation in the presence of PSGAG is consistent with the findings of Glade et al. who found decreased cellular proliferation in chondrocyte cultures[25]. Our finding suggest a similar inhibition of proliferation when MSCs are exposed to PSGAG. Concentrations of PGE_2 appeared decreased in PSGAG and SF treated media supernatants, though differences were not statistically significant. The possibly decreased mediator production in MSCs expanded with these test solutions supports our hypothesis of cell death in PSGAG cultures and osteogenic differentiation in SF cultures, however, additional study is warranted. The detrimental effects of PSGAG on MSC viability could be density dependent, as the CFU-F plates were plated at 18 cells/cm^2 and showed the most dramatic effect with failure to form colonies. It is possible that higher densities allowed MSCs to acclimate or somewhat recover from the effects of PSGAG in the trilineage differentiation and immunophenotyping assays, which used cells plated at 10,000 cells/cm^2 for expansion.

Though the immunophenotypic profile of $CD29^{+}$, $CD45^{-}$, and $MHCII^{-}$ was retained in PSGAG and SF cultures, PSGAG cultures appeared to have reduced expression of CD44 compared to controls and other test solution cultures. MSCs have previously demonstrated a range of expression for CD29, positive expression of CD44 (> 70%), and robustly negative expression of MHCII ($\leq$ 5.0%)[73]. Possibly decreased expression of CD44 in PSGAG cultures could result in decreased adhesion and proliferation[74]. Additional investigation with a greater sample size is warranted.

MSCs have demonstrated a fluctuating expression of MHCII and other surface proteins when primed by exposure to cytokines and chemokines in an inflamed environment, including those present in synovial fluid of a diseased joint[39,58]. A significant increase in MHCII expression was not elicited by exposure to any conditions in the current study, including SF. However, SF cultures demonstrated greater reduction in proliferation than PSGAG cultures. This is in contrast to the results of the pilot study, which suggested a possible increase in proliferation. This discrepancy could be due to the difference in synovial fluid used for each experiment. For the pilot study, pooled synovial fluid was used, while in the current study, synovial fluid from nly two horses was used. Individual differences from the two synovial fluid donor horses could be the reason for the discrepancy between our two studies. Nevertheless, replacement of endogenous SF with HA before MSC administration could be beneficial to the survival of MSC once injected into the joint, and may delay priming MSCs, decreasing the risk of an immune response after injection[39].

All test solution groups retained the ability to differentiate into adipocytes, chondrocytes, and osteoblasts after 120 hours of expansion with test solutions. Chondrogenesis of canine bone marrow-derived MSCs in the presence of PSGAG has been evaluated previously in alginate and micromass cultures[75]. In micromass cultures with PSGAG, chondrogenesis was inhibited, as decreased proteoglycan production was observed by Alcian blue staining[75]. Results of the current study suggest a possible reduction in chondrogenic ability after MSC expansion with PSGAG as well. Though chondrogenic differentiation was successful, MSCs cultured with PSGAG appeared to produce less ECM compared to other treatments, according to the scoring of distance between cells. This possibility is in agreement with results from the previous study[75].

Limitations of the current study include a low sample size (n = 6), testing few cellular densities, and only testing one concentration per test solution. Multiple cell densities for intra-articular injection have been studied in the horse and other species such as humans and rabbits, the most popular doses ranging from 10^6 to 10^8 cells[44,76]. Therefore, expanding MSCs at additional densities would have provided more information on the apparent density-dependent effects of HA and PSGAG on MSC viability. Further, intra-articular dosing varies with joint size, so testing a clinically relevant range of concentrations for each test solution would have also provided more comprehensive information. Additionally, testing for the presence of other inflammatory mediators such as interleukin-10 (IL-10) or IL-6 would provide more information on immunomodulatory functions of MSCs following expansion with these medications.

Results of the current study reveal that MSC expansion with PSGAG or SF will hinder viability and proliferation, while expansion with HA may increase viability without hindering proliferation *in vitro*. Therefore, combination of MSCs with PSGAG and SF may inhibit viability and function of implanted MSCs, while combination of MSCs with HA may supplement proliferation and immunomodulatory function of MSCs in culture and after intra-articular implantation while providing the anti-inflammatory and disease modifying effects demonstrated by HA in other studies[14]. *In vivo* studies are warranted to investigate these possibilities.

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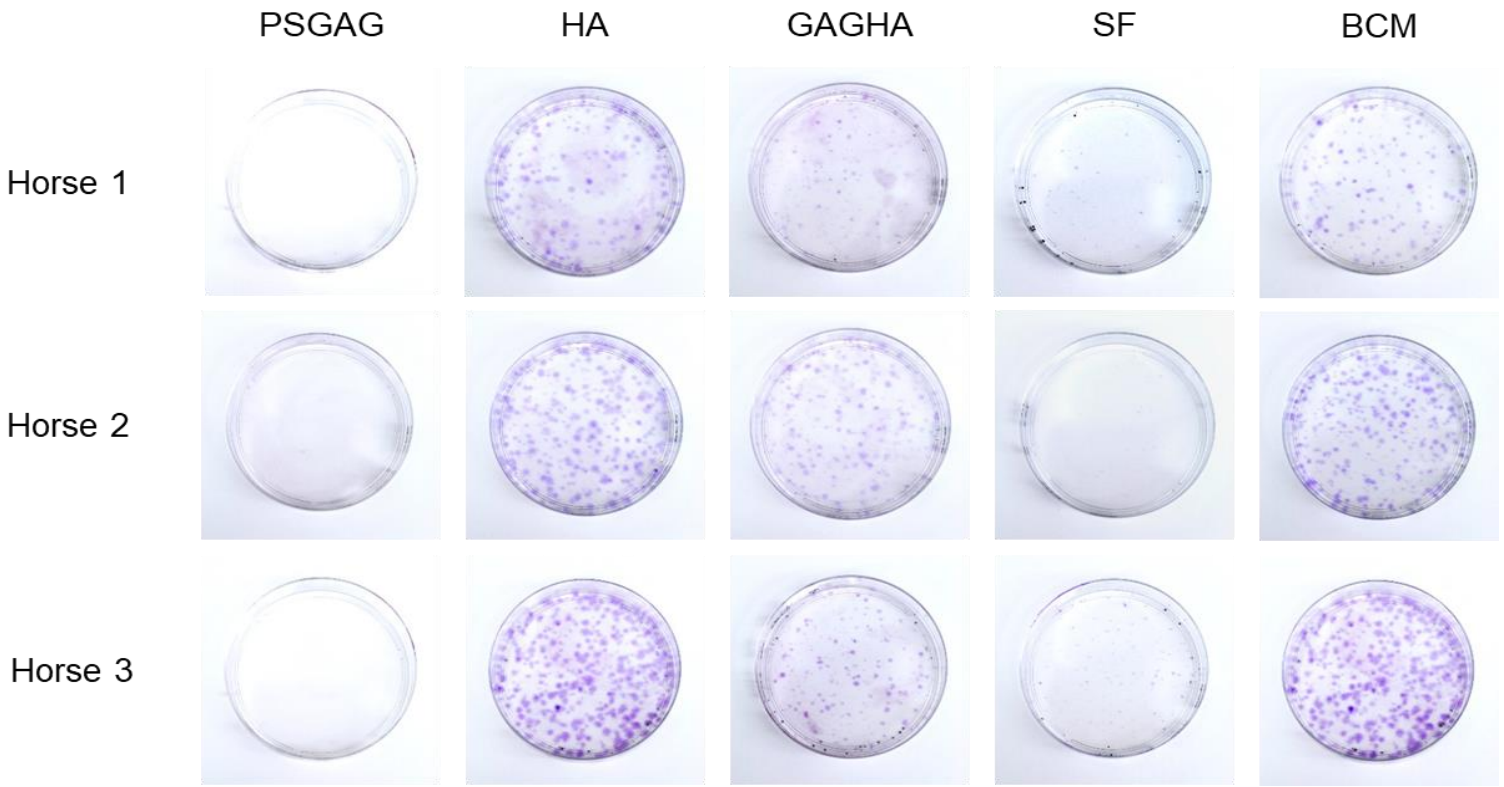
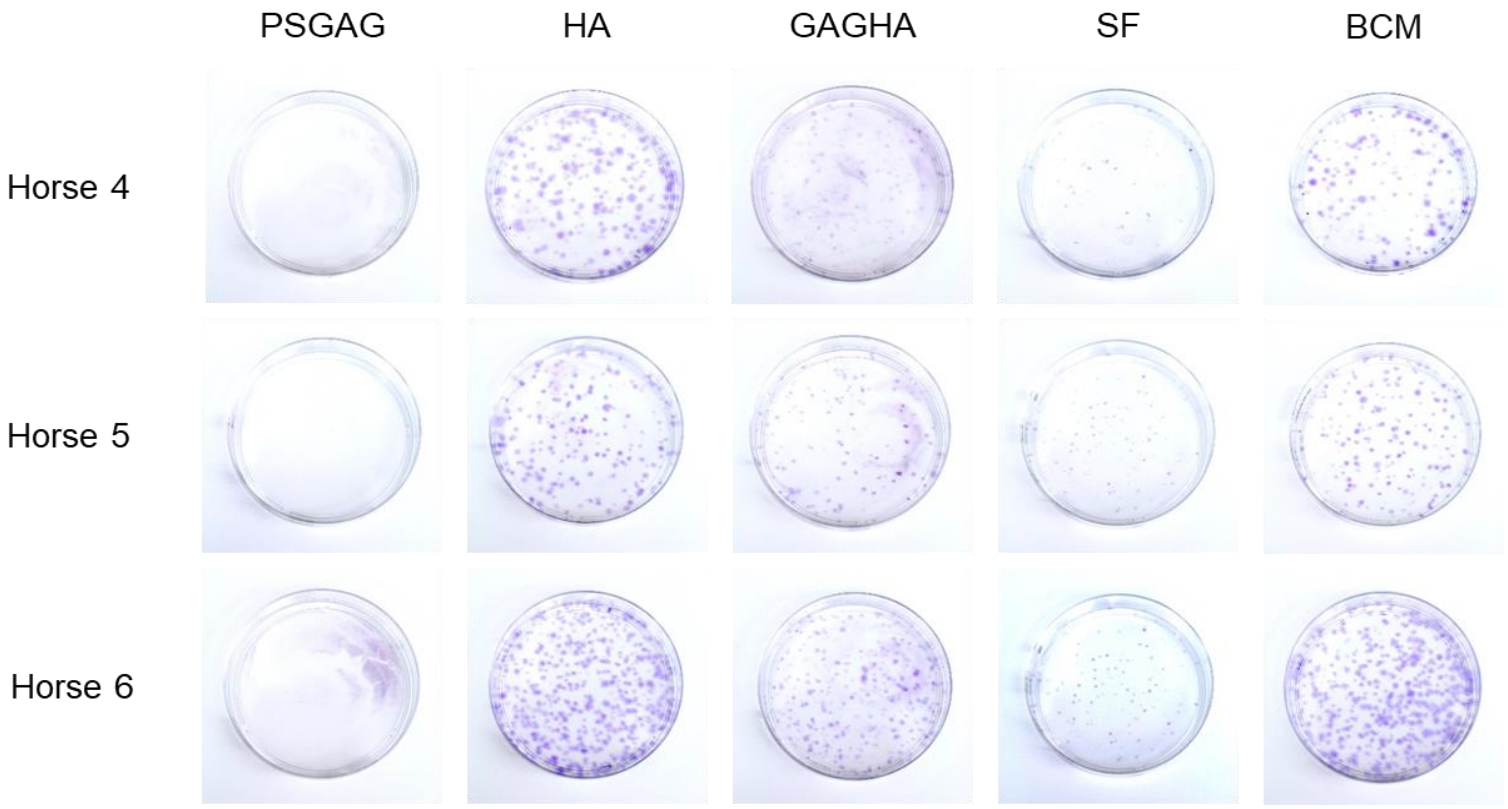
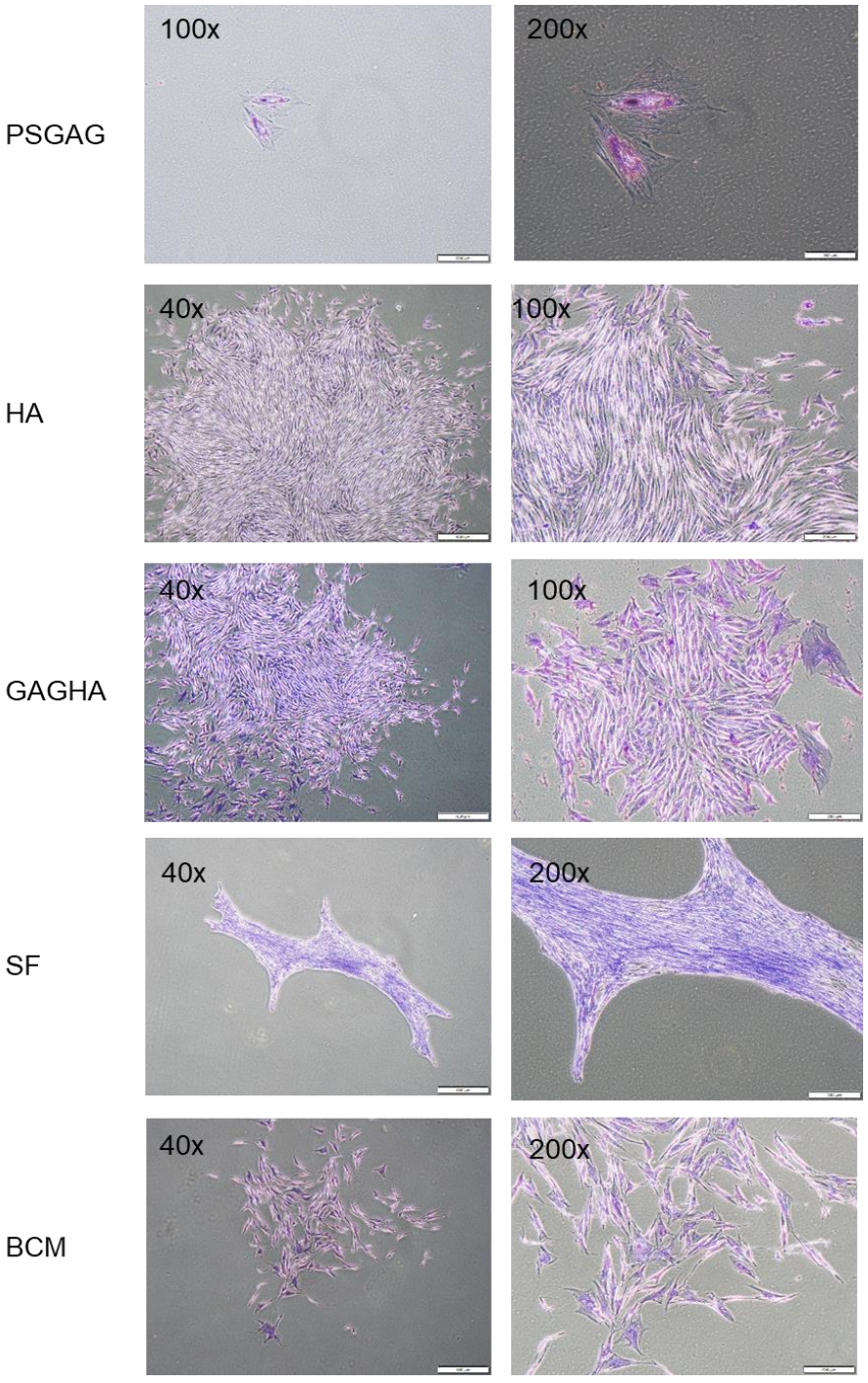
APPENDIX A



Colony Forming Unit 10 cm Plates, Horses 1 – 3

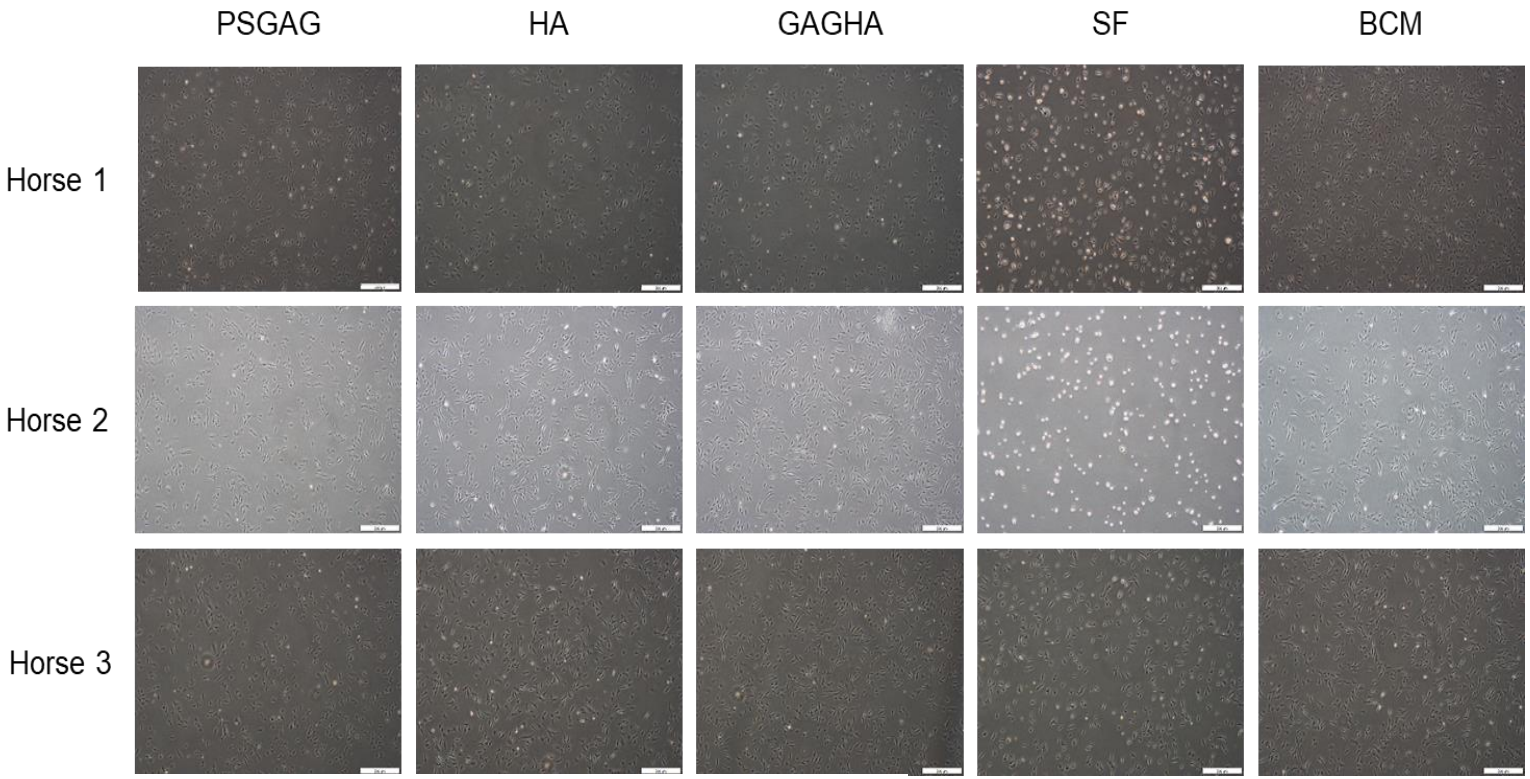


Colony Forming Unit 10 cm Plates, Horses 4 – 6

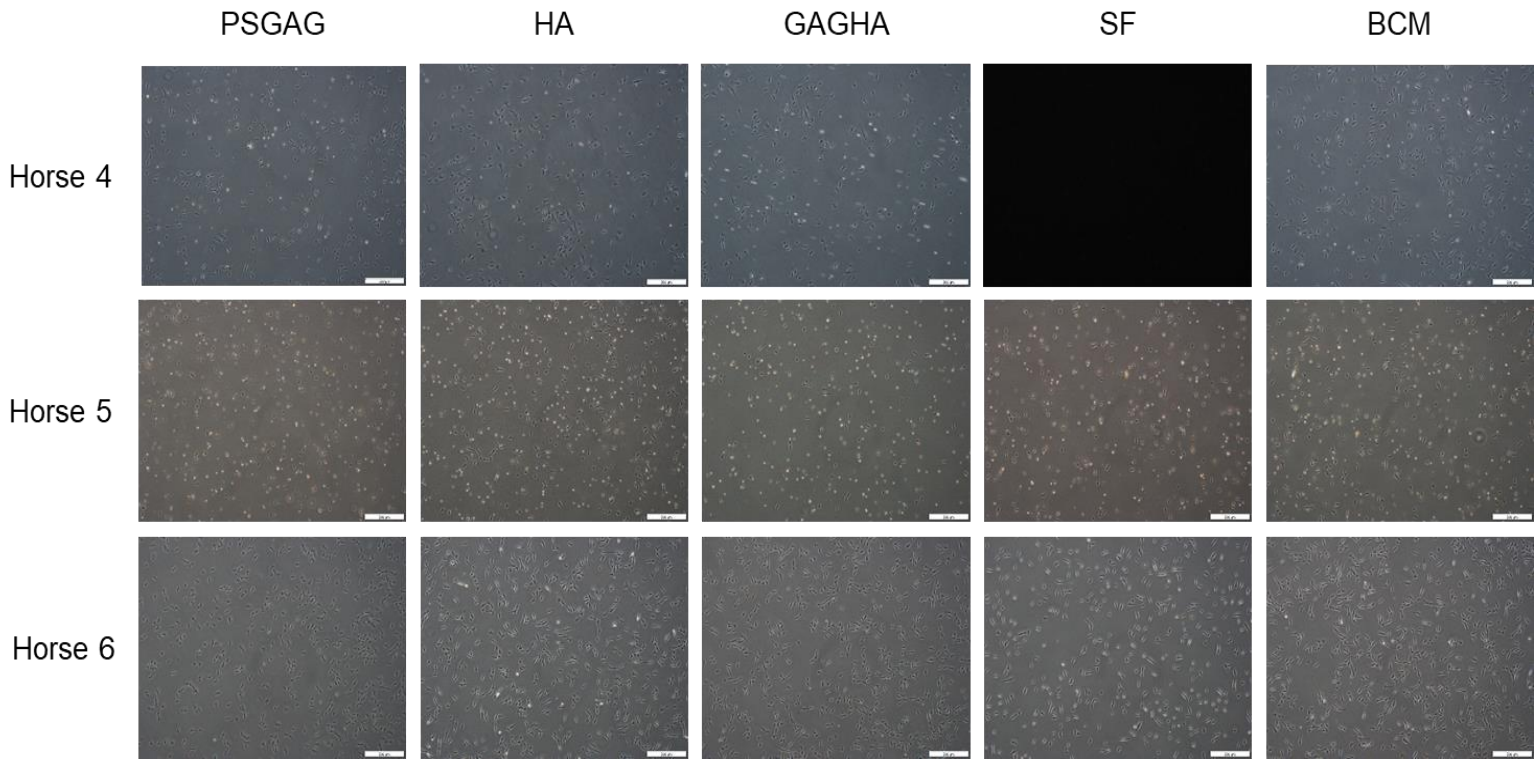


Representative Colony Forming Unit Microscopic Images

APPENDIX B



24 Hour Culture Evaluation Images, 40x, Horses 1 – 3



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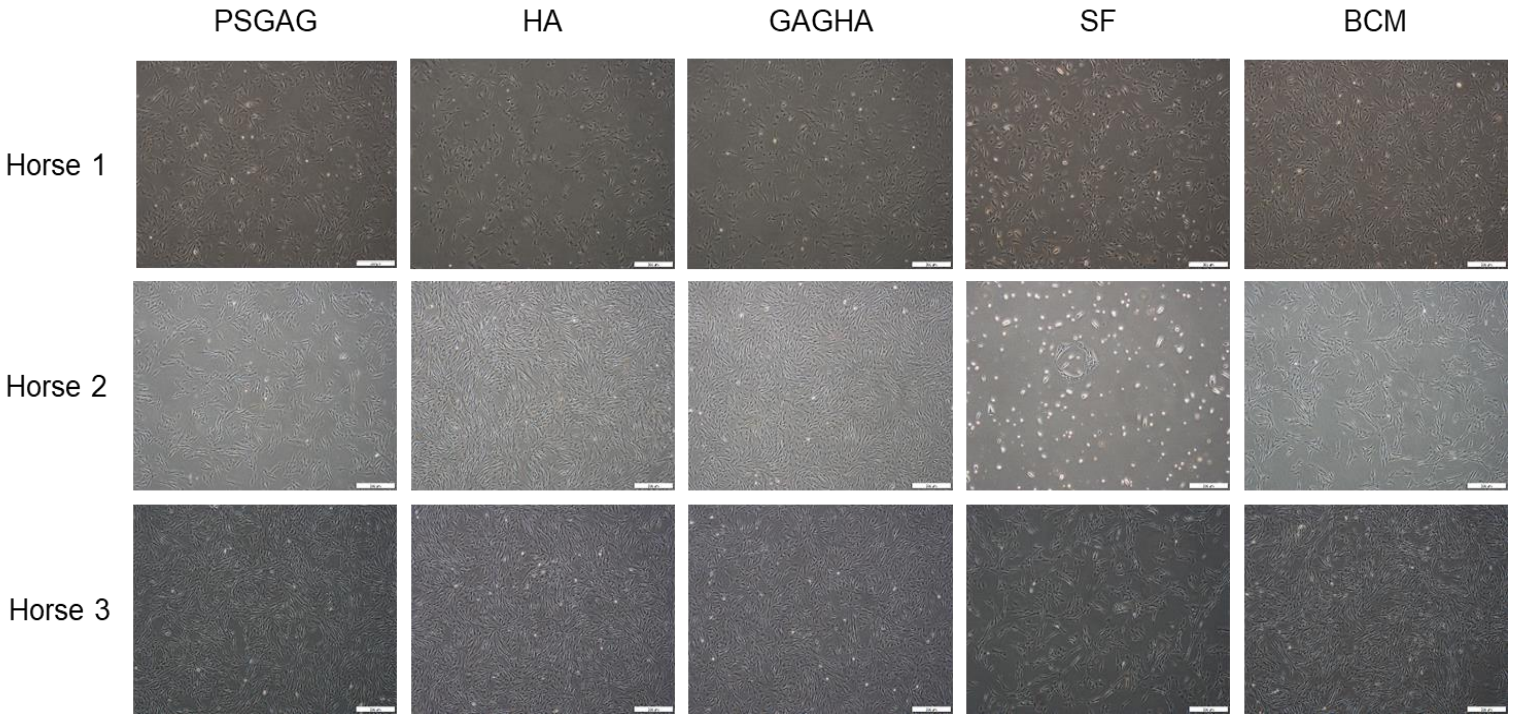
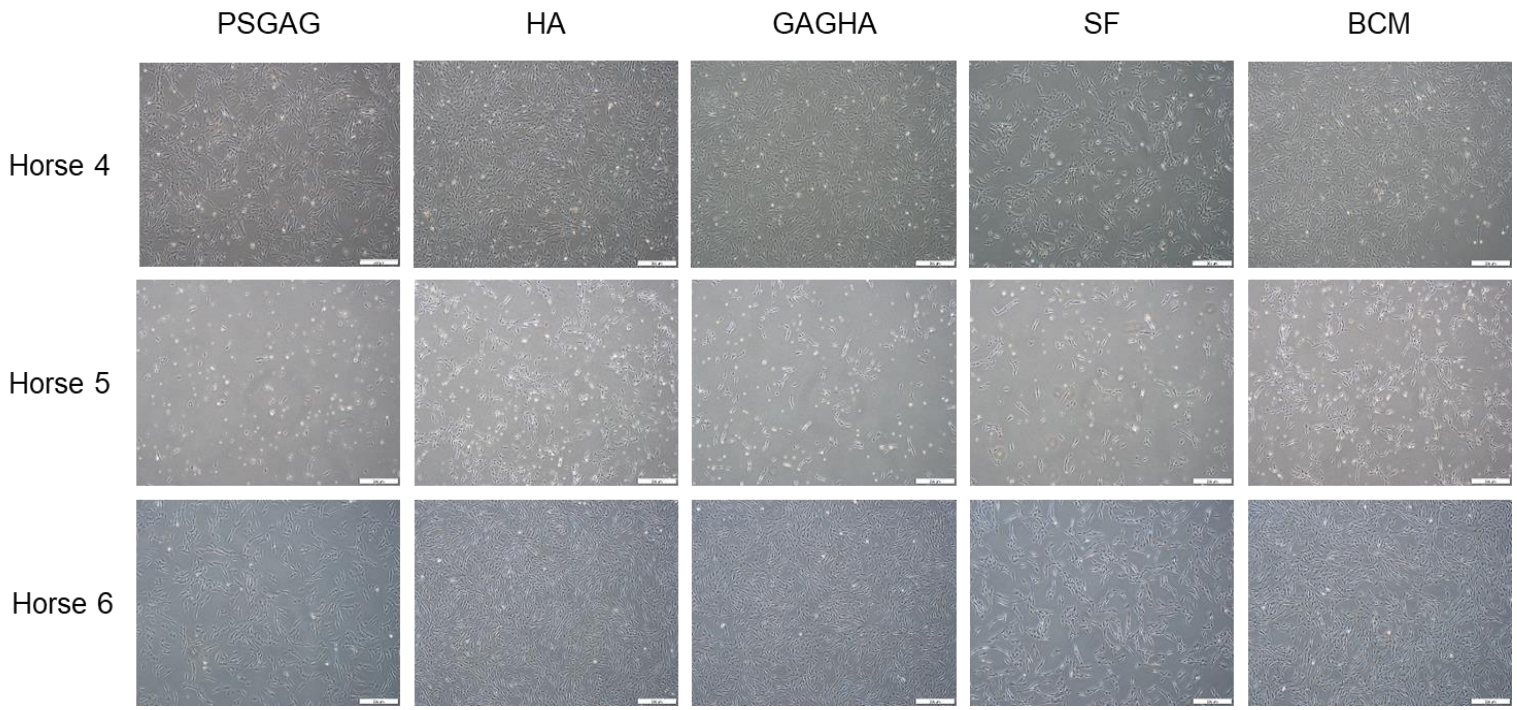
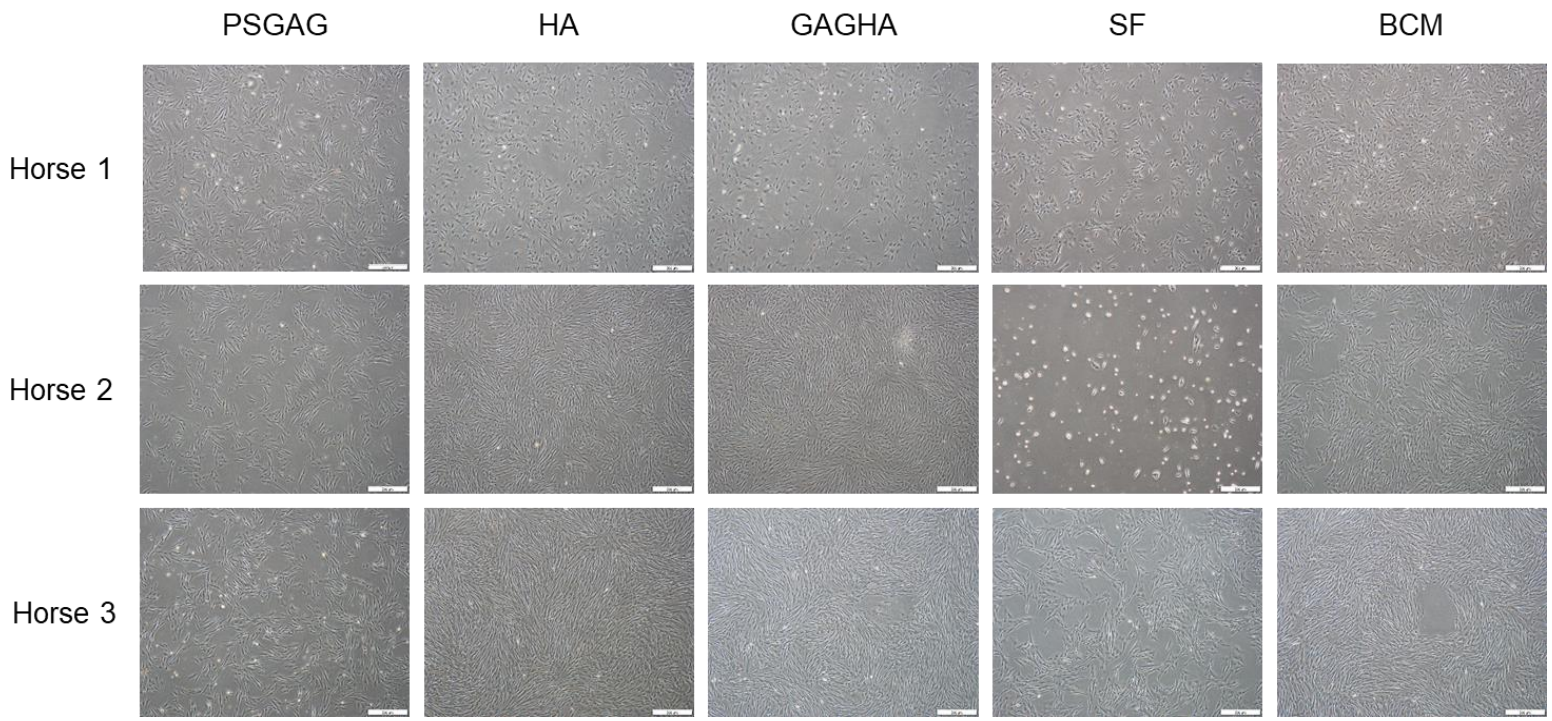


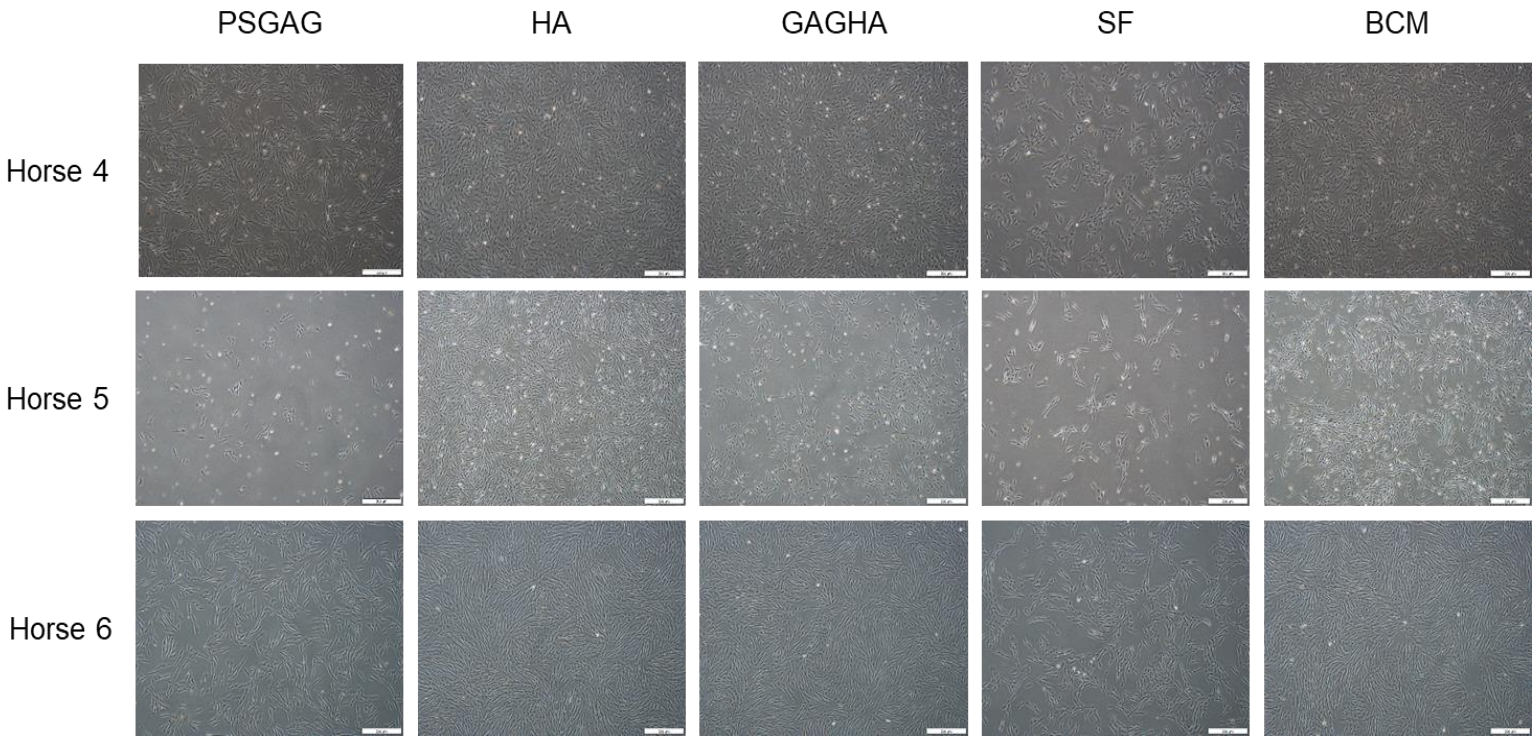
72 Hour Culture Evaluation Images, 40x, Horses 1 - 3



72 Hour Culture Evaluation Images, 40x, Horses 4 – 6

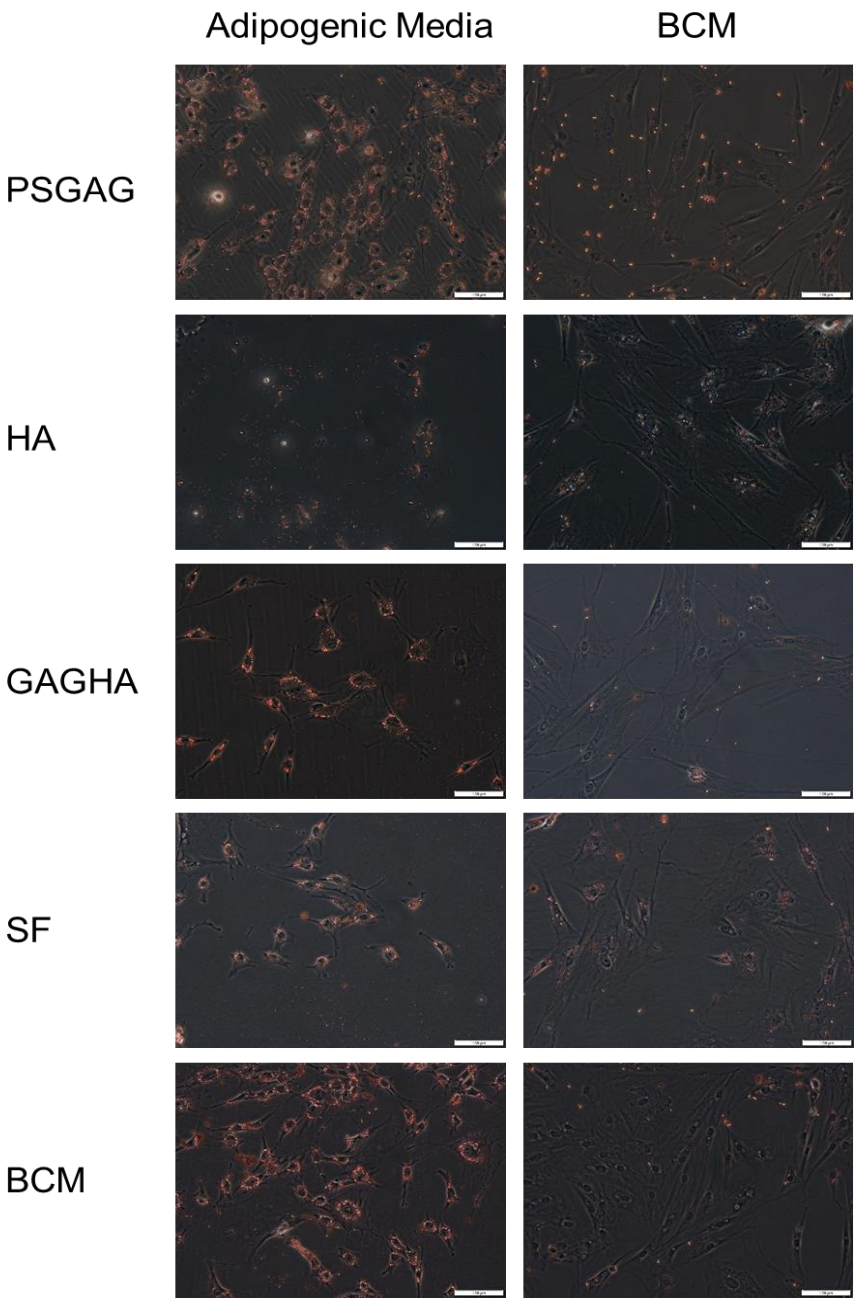


120 Hour Culture Evaluation Images, 40x, Horses 1 – 3

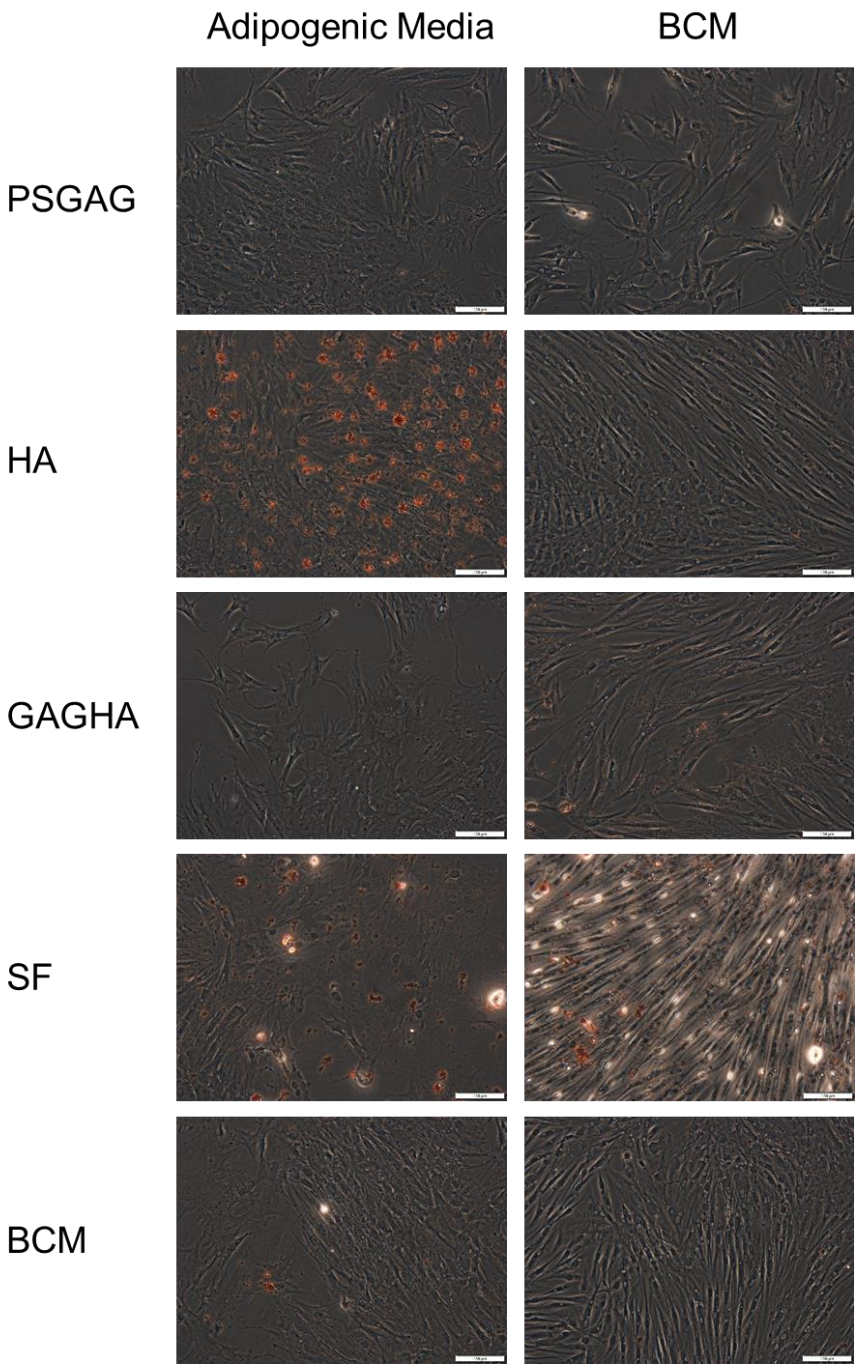


120 Hour Culture Evaluation Images, 40x, Horses 4 – 6

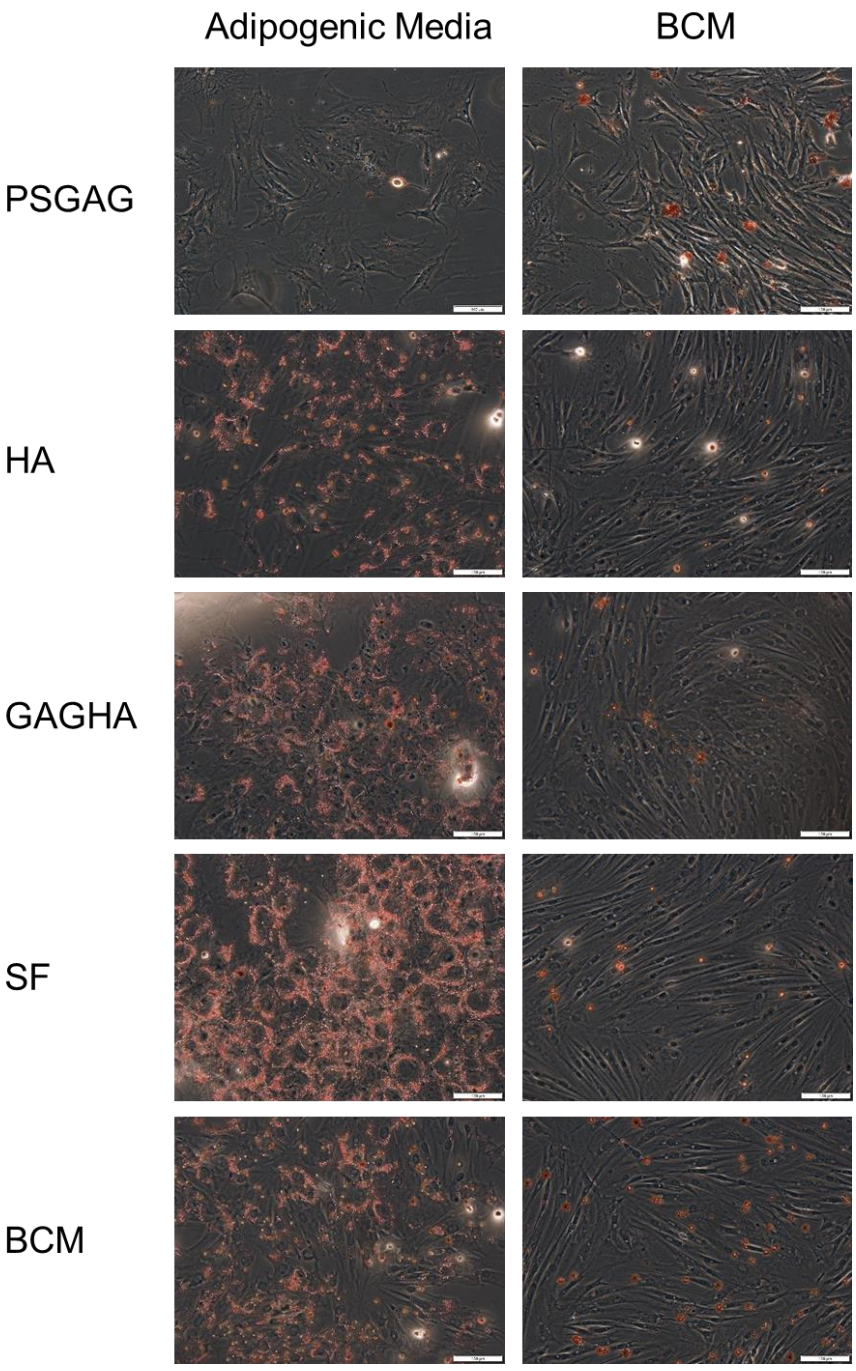
APPENDIX C



Representative Adipogenesis Images, Horse 1



Representative Adipogenesis Images, Horse 2



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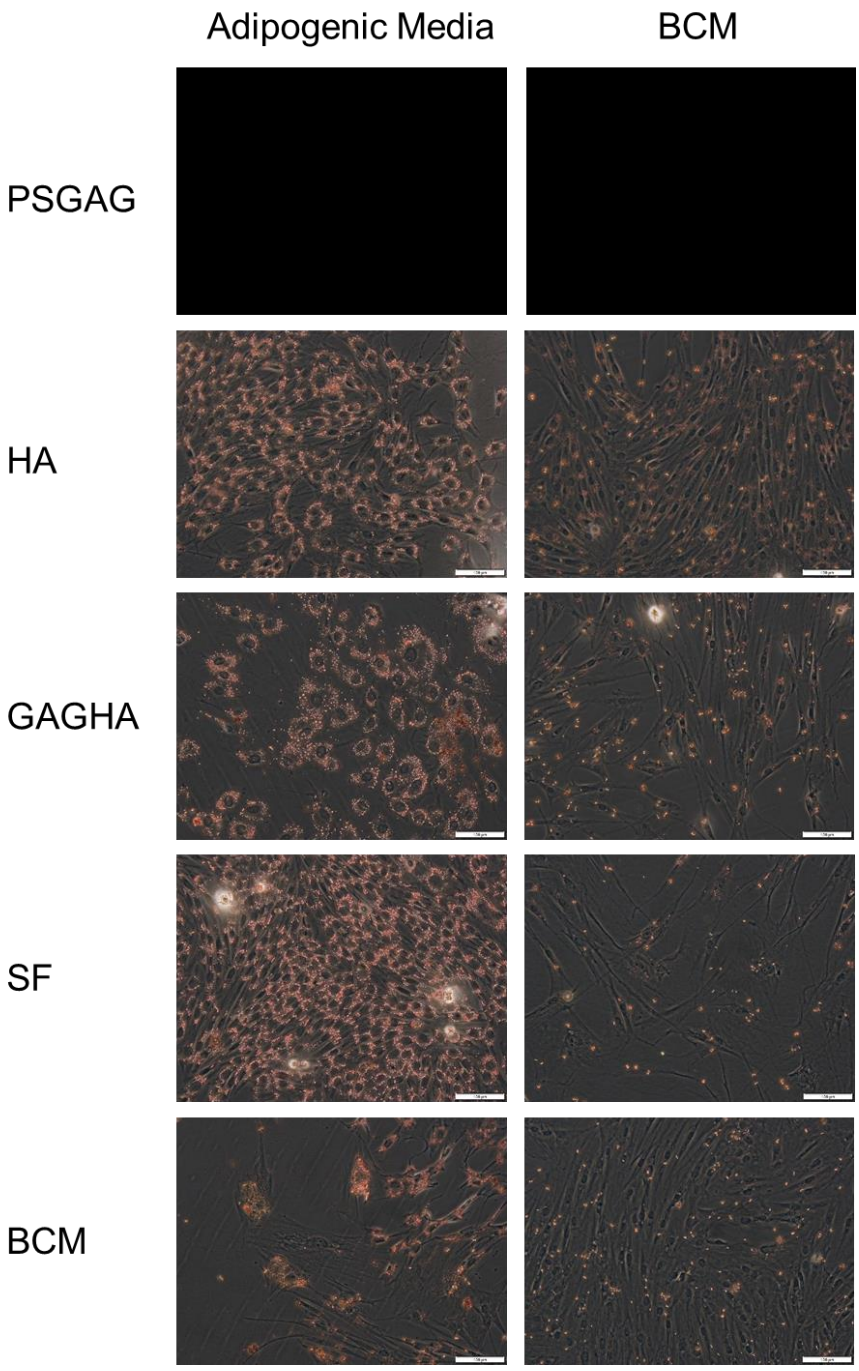
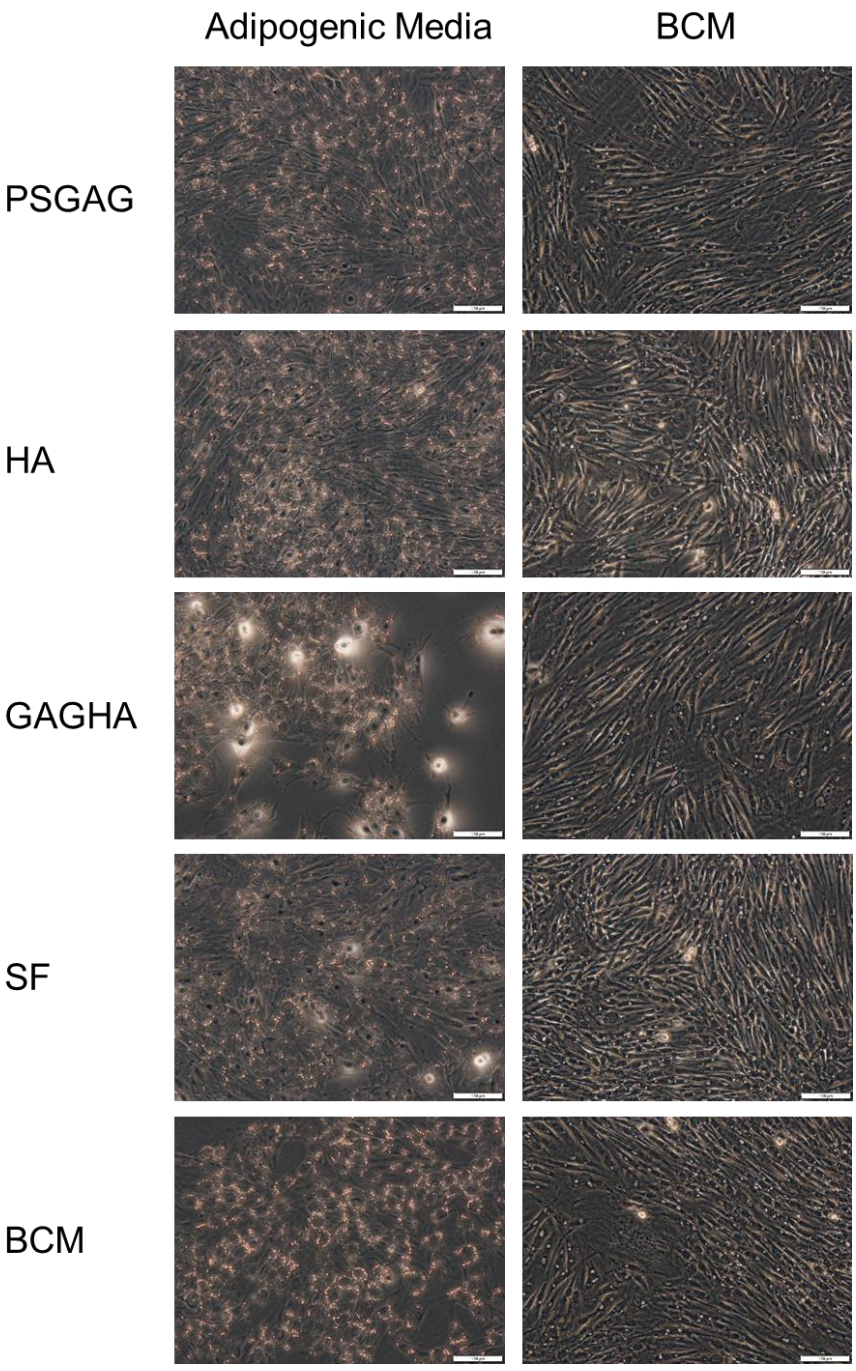


Representative Adipogenesis Images, Horse 4



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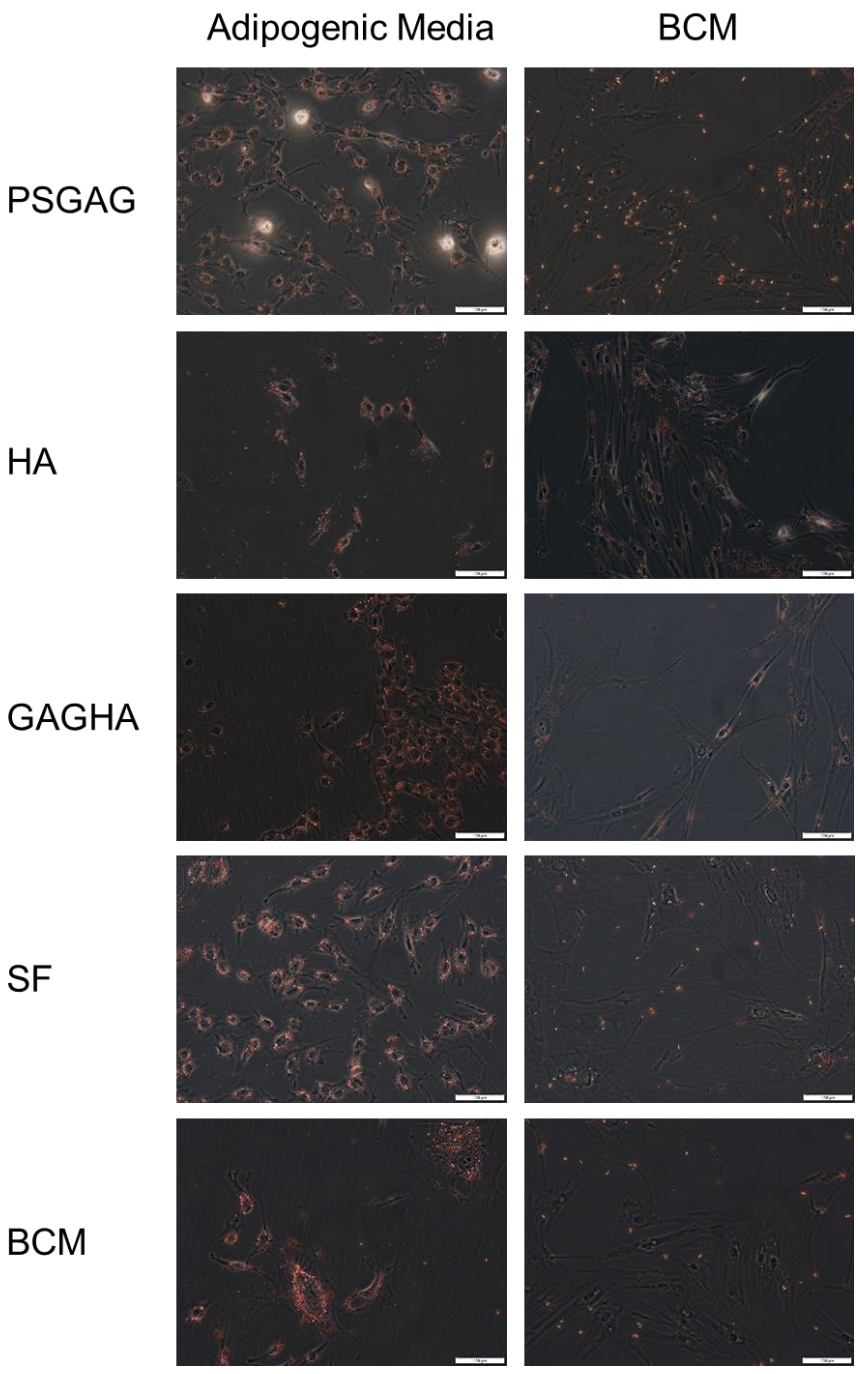
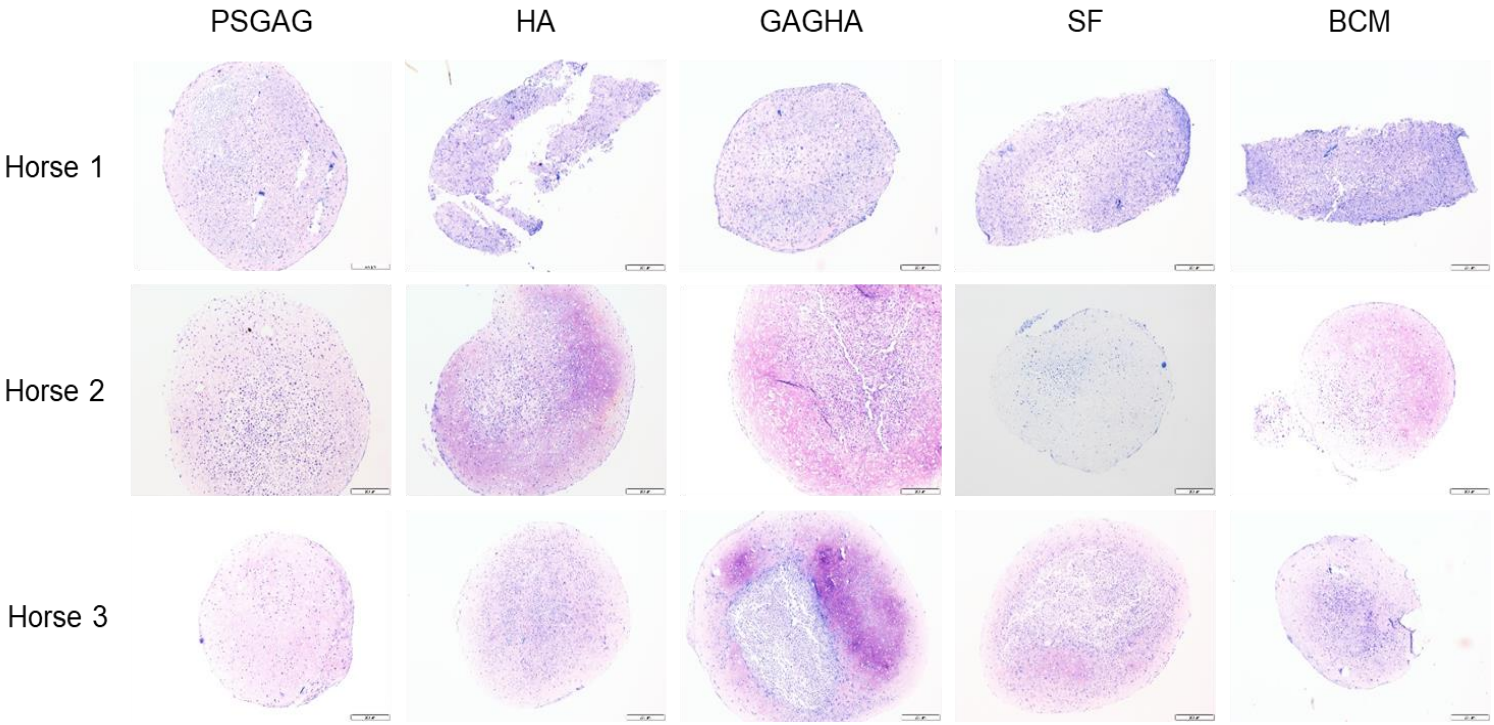
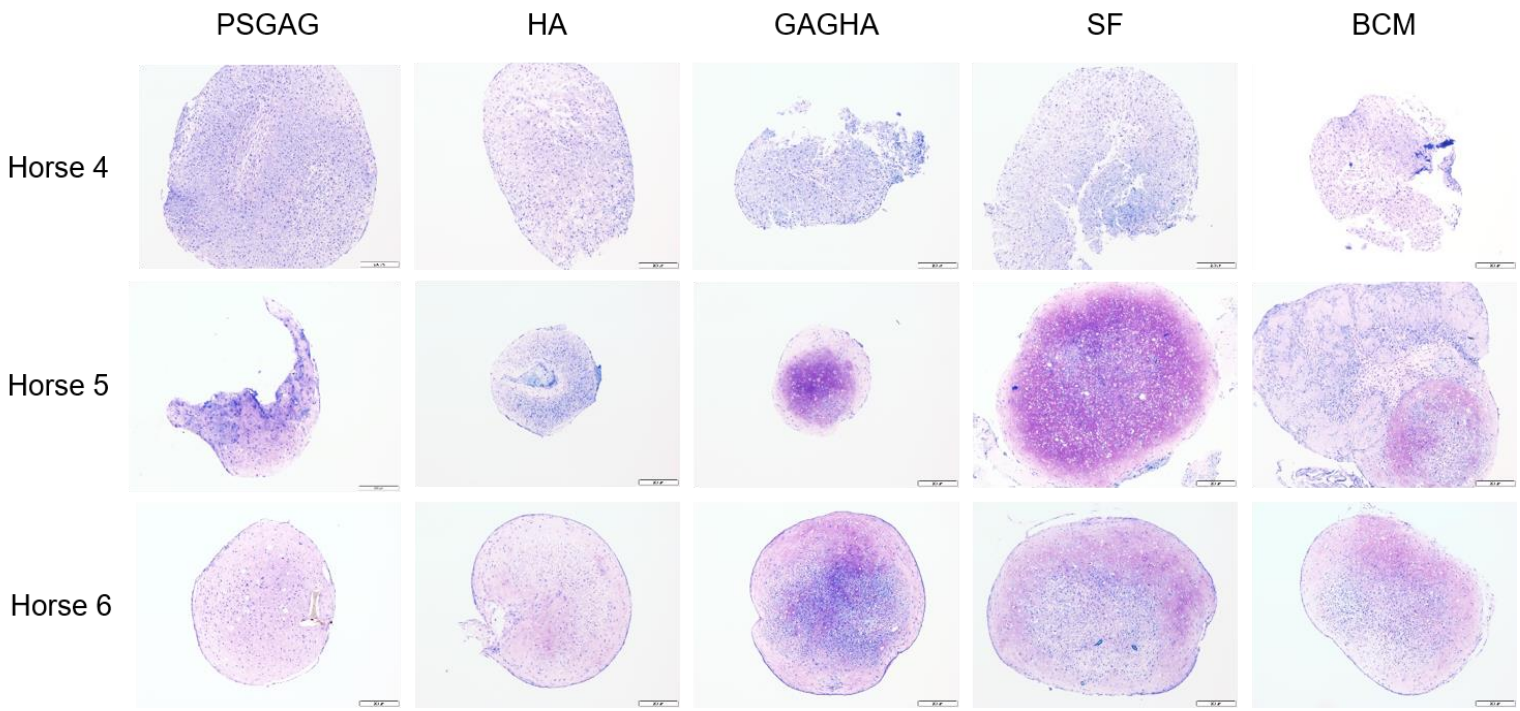


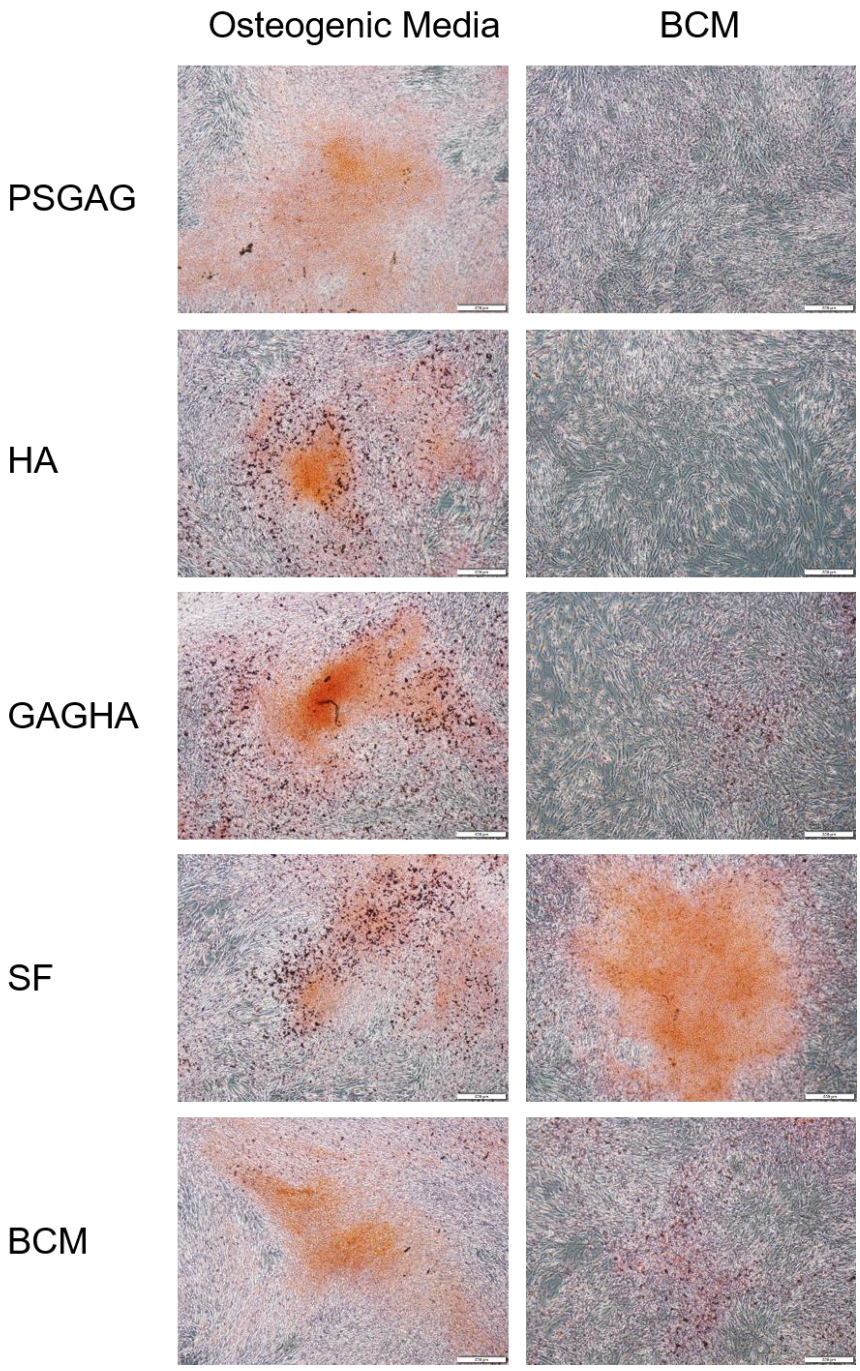
Representative Adipogenesis Images, Horse 6



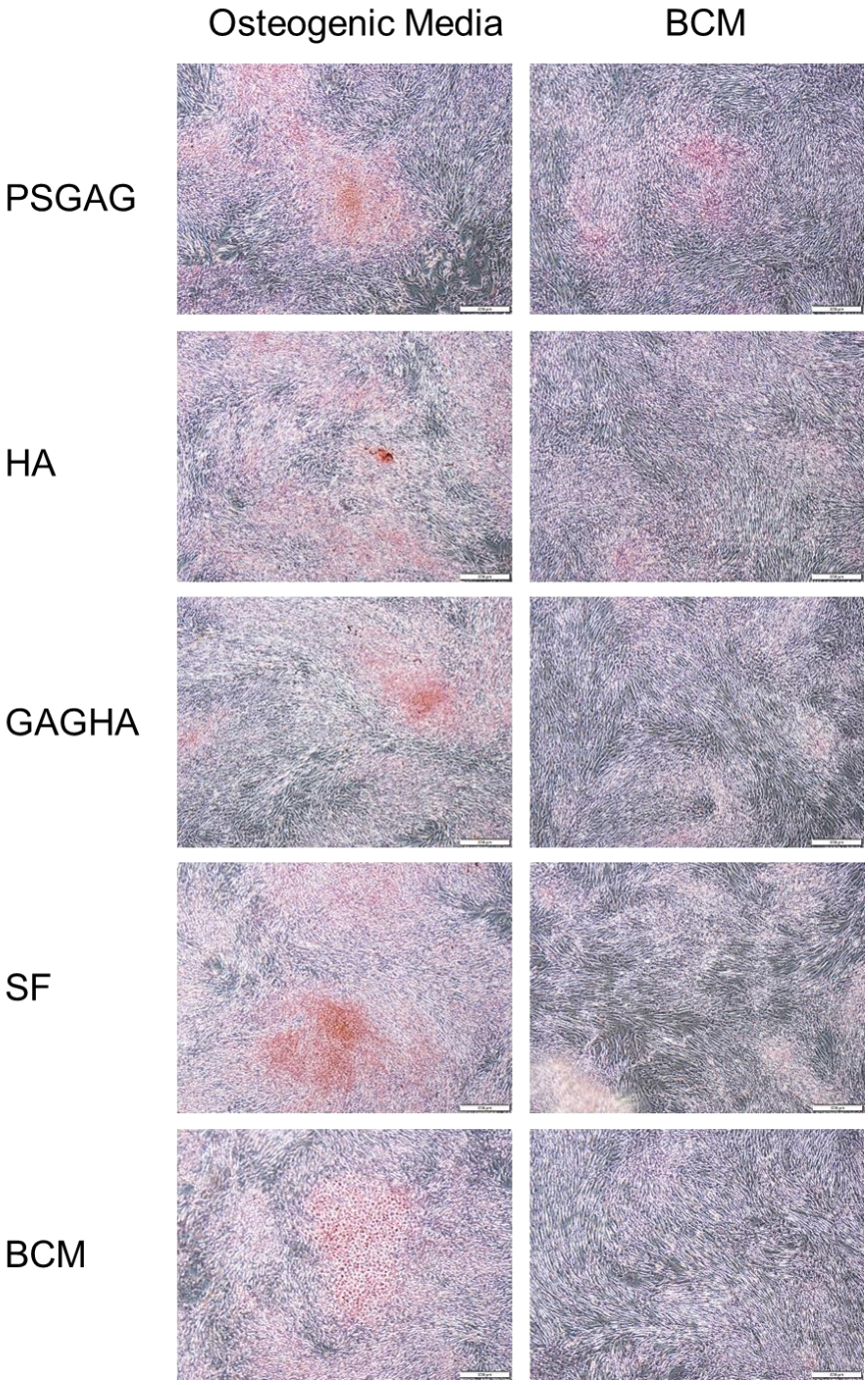
Representative Chondrogenesis Images, Horses 1 - 3



Representative Chondrogenesis Images, Horses 4 – 6



Representative Osteogenesis Images, Horse 1



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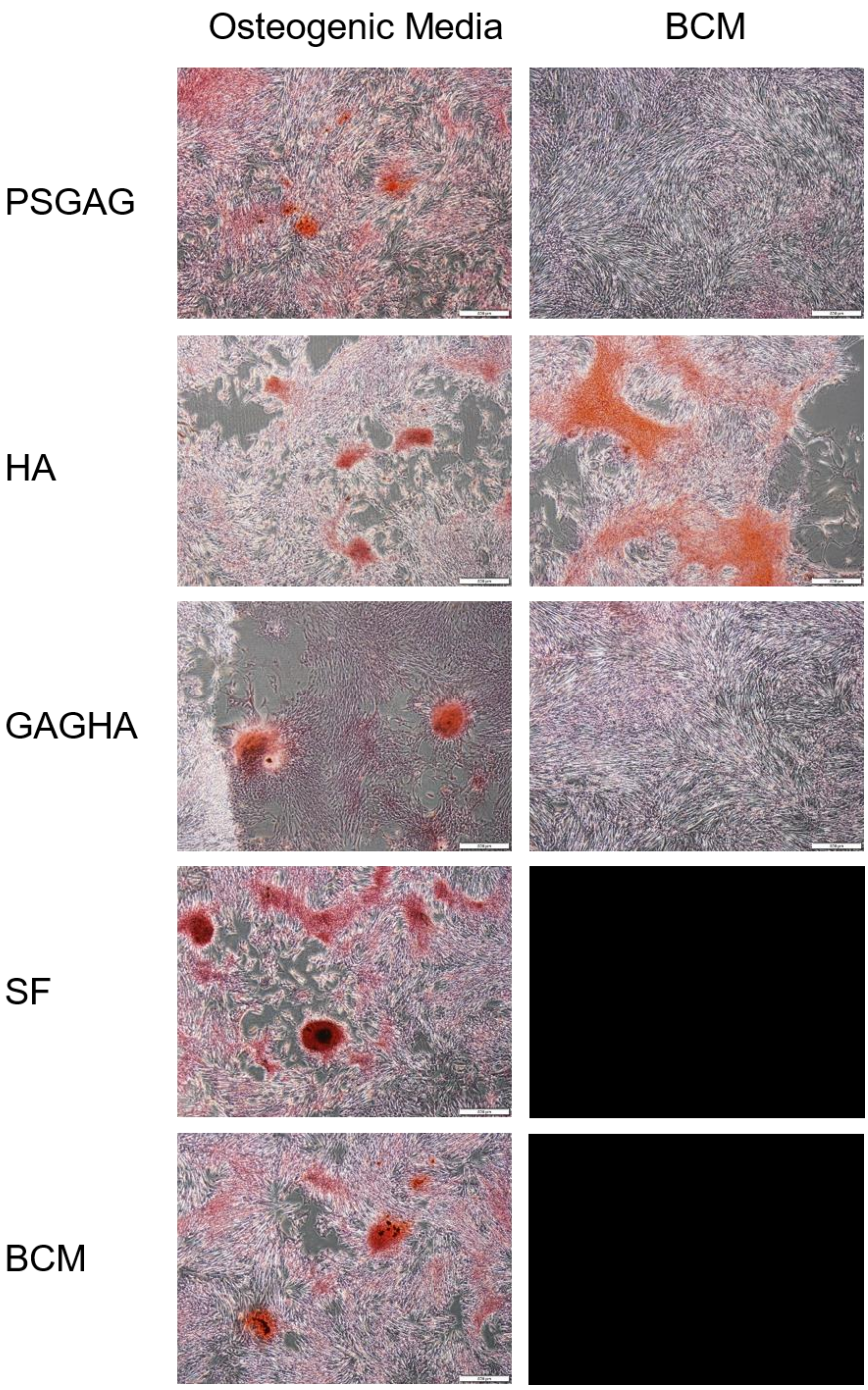

Representative Osteogenesis Images, Horse 3
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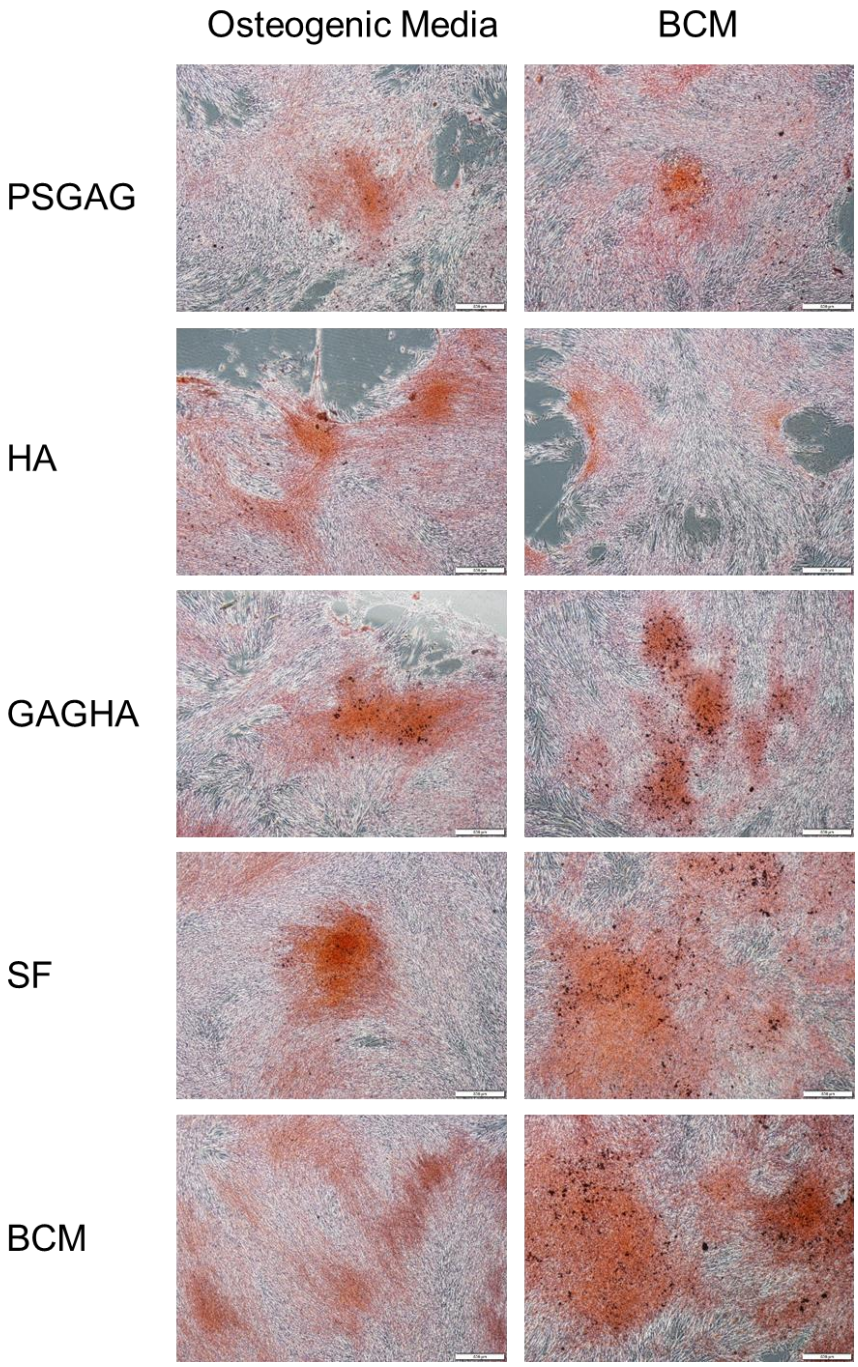

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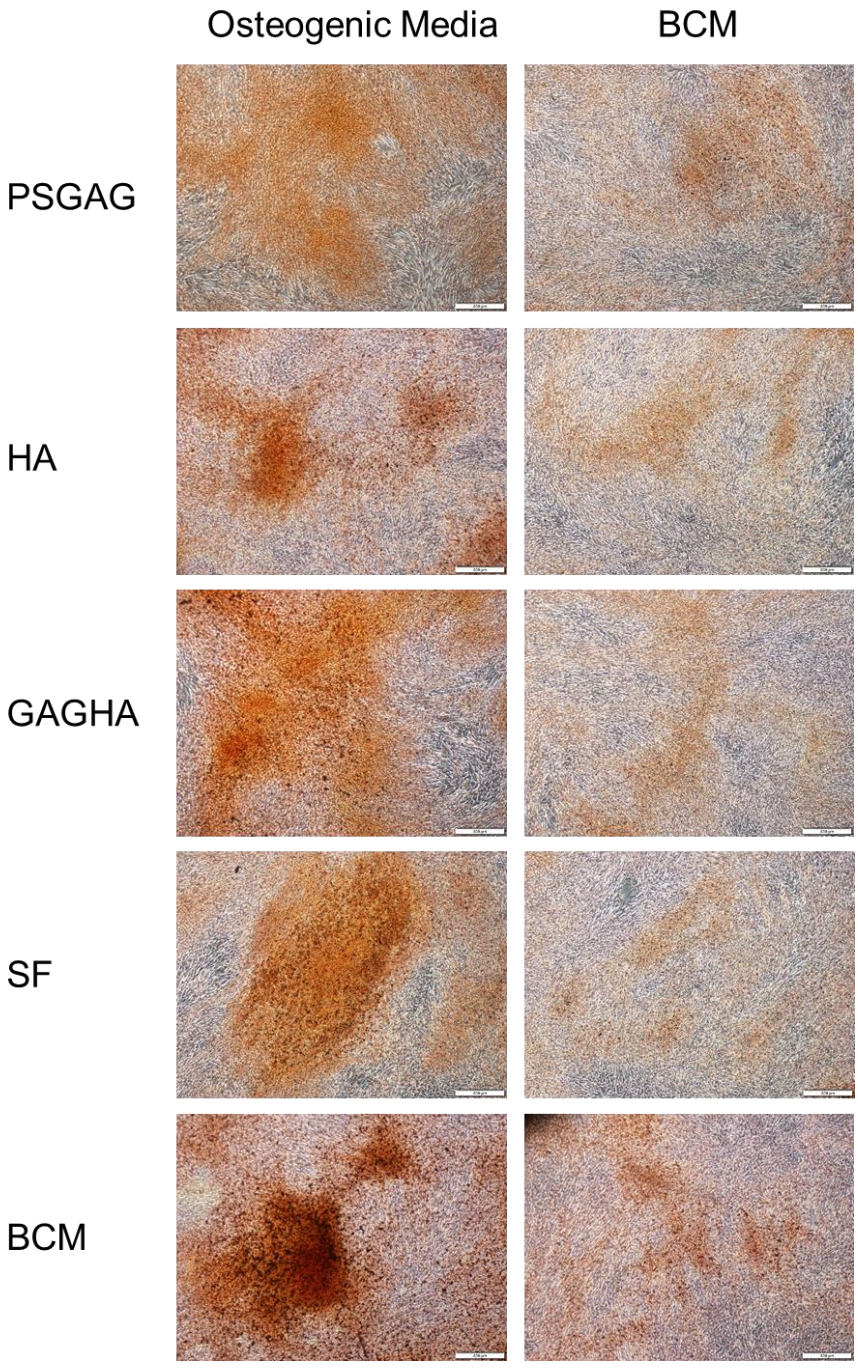

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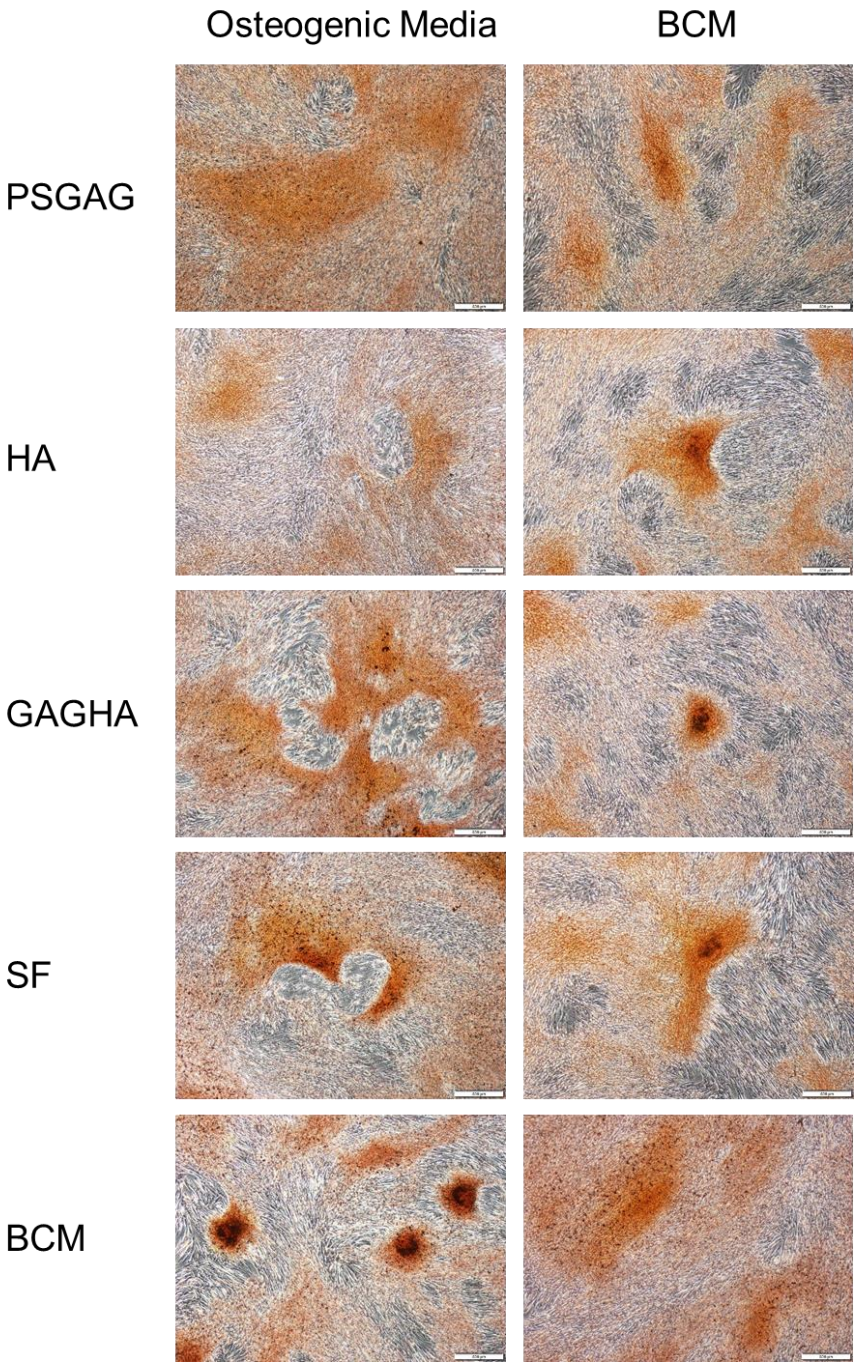

Representative Osteogenesis Images, Horse 6