Equine Endothelial Colony-Forming Cells: Phenotypic Enhancement and *Ex vivo* Angiogenesis Studies

by

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Abstract

Ischemic diseases in horses are common and often life-threatening, thus cell-based therapies to improve angiogenesis are gaining interest. Endothelial colony-forming cells (ECFCs) can form *de novo* blood vessels in areas of ischemia and have been isolated from peripheral blood of horses; however, there is the potential of having a mixed cell population rather than pure ECFCs. Selection of ECFCs with the endothelial cell (EC) phenotype and enhancing the endothelial pathway in culture may improve angiogenesis. Angiogenesis and the use of alternative sources of growth factors can be investigated *ex vivo* to better mimic the processes seen *in vivo*. The study objectives were to isolate ECFCs with an EC phenotype using cell sorting and to enhance expression of the EC pathway in culture by the addition of the nitric oxide (NO) donor S-nitroso cysteine (CysNO). To optimize media for EC phenotype, the effects of equine platelet lysate (ePL) were evaluated through the arterial ring assay.

Equine ECFCs were sorted by fluorescence- (FACS) or magnetic-activated cell sorting (MACS) based on acetylated low-density lipoprotein uptake or expression of cluster of differentiation (CD) 31. Sorted cells were characterized based on phenotype and *in vitro* angiogenesis. To evaluate the effects of NO, CysNO was added to the endothelial growth media (EGM) of cultured ECFCs, and cells were evaluated for CD31 expression, viability, growth and tubule formation *in vitro*. The angiogenic effect of ePL was evaluated by supplementing equine arterial rings with EGM-containing ePL.

Equine ECFCs were successfully sorted by FACS and MACS, but once expanded, their phenotype and function were comparable to non-sorted ECFCs. The addition of CysNO had no effect in ECFC function *in vitro*, viability, or phenotype. Equine arterial rings served as an *ex vivo* model of angiogenesis, and although ePL was not superior to horse serum, it was successful in supporting angiogenesis.

Using appropriate protocols allows for the sorting of equine ECFCs, and both ePL and PPP, similarly to HS, have the potential to be used for the culture of ECFCs. However, future research studying the use of alternative EC surface markers or refined colony selection is needed to confirm identity and reduce the mixed population of cells isolated with current methods.

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List of Abbreviations

Ac-LDL	Acetylated low-density lipoprotein
Akt	Protein kinase B
ANOVA	Analysis of variance
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CH	Number of cells
CPDL	Cumulative population doubling level
CS	Number of cells seeded
CysNO	S-nitroso cysteine
DGC	Density gradient centrifugation
EBM	Endothelial basal media
EC	Endothelial cells
ECFCs	Endothelial colony-forming cells
EDTA	Ethylenediaminetetraacetic acid
EGM	Endothelial growth media
ELISA	Enzyme-linked immunosorbent assay

eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells
ePL	Equine platelet lysate
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FC	Flow cytometry
FS	First sprout
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HIF-1α	Hypoxia-inducible factor-1α
HS	Horse serum
HUVEC	Human umbilical vein endothelial cells
IGF-1	Insulin growth factor-1
IL	Interleukin
IU	International units
IV	Intravenously
L-NAME	L-N^G-Nitro arginine methyl ester
MACS	Magnetic activated cell sorting

ML	Matrigel lysis
MNC	Mononuclear cells
MNG	Maximum network growth
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
NCD	Number of cell doublings
NO	Nitric oxide
NOS	Nitric oxide synthase
P	Passage
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer solution
PDGF-BB	Platelet-derived growth factor BB
PDT	Population doubling time
PI3K	Phosphoinositide 3-kinase
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
SDF-1	Stromal cell-derived factor-1

SNAP	S-nitroso-N-acetylpenicillamine
TGF-β	Transforming growth factor-β
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VNA	Vascular network area
VR	Vascular regression
vWF	von Willebrand factor
WBC	White blood cell

Chapter 1 – Literature review

Impaired angiogenesis in horses can be the result of common ischemic conditions such as laminitis, delayed wound healing or some types of colic, thus cellular therapies using endothelial progenitor cells (EPCs) have been investigated due to its potential to both stimulate blood vessel formation and repair. To understand the possibilities of regenerative angiogenesis as a future treatment in horses, the following review will focus on discussing the origin of the progenitor cells responsible for angiogenesis, and how different stimuli can drive them towards the formation of new blood vessel. The current challenges in isolation and grand scale expansion of EPCs makes its clinical use difficult; therefore, modifications in isolation and cell culture protocols to overcome these challenges, and the different assays to evaluate its angiogenic function are discussed.

1.1 Blood vessel formation

Fully functional blood vessels are formed by the interaction of two cell types, endothelial cells (ECs) and perivascular cells. Endothelial cells organize in a monolayer to form the inner lining of the vessel wall, and are considered to be very versatile and multifunctional. The endothelium is a semipermeable barrier that allows for transfer of molecules, thus being responsible for metabolic and synthetic functions by close communication with the underlying perivascular cells.[1] Perivascular cells refer to pericytes, vascular smooth muscle cells or mural cells; they will coat the surface of the vascular tubule, and in addition to providing support to

the ECs, they also aid in regulation of blood flow, and by direct contact and paracrine signaling, can enable exchange of ions and small molecules.[2]

The two main mechanisms of blood vessel formation are angiogenesis and vasculogenesis. Angiogenesis is the formation of new blood vessels that branch off from existing vessels and is often referred as adult neovascularization. On the other hand, vasculogenesis refers to the formation of new blood vessels from endothelial precursors that differentiate *in situ*, either in areas that previously had no vascular networks or in tissues that have experienced trauma.[3-5] Vasculogenesis is critical during embryogenesis for blood vessel formation, however, the discovery of endothelial progenitor cells (EPCs) in adults suggests that vasculogenesis may occur throughout adult life.[5]

Angiogenesis is initially stimulated by substances such as nitric oxide (NO), vascular endothelial growth factor (VEGF) and fibroblast growth factor. During vessel sprouting, these angiogenic factors will stimulate ECs, which in turn will secrete proteases to degrade the extracellular matrix. This process will allow the ECs to invade and form a new vascular network.[2, 5] During sprouting angiogenesis, migrating ECs form simple vascular tubes, which are then remodeled by the influence of blood flow and interaction with perivascular cells, leading to the formation of mature and fully functional vasculature. These cellular processes are stimulated by different proangiogenic signaling pathways, such as VEGF, which regulates both cell division and branching. VEGF-A binds two high affinity cell surface receptors on ECs, vascular endothelial growth factor receptor (VEGFR)-2 and VEGFR-1. The amount of available VEGF regulates the rate of EC division, and the presence of a gradient concentration and shear stress will lead the sprouting process.[6] Vascular ECs have limited regenerative capacity,

making EPCs a promising treatment option due to their ability to maintain endothelial integrity, function, and postnatal neovascularization.[7]

1.2 Endothelial progenitor cells

During embryogenesis, blood vessel formation starts with the appearance of the hemangioblast, which is derived from the mesoderm. Further remodeling of this early vasculature leads to the formation of a functional vascular network.[3] However, due to the close association between hematopoietic and ECs during embryogenesis, there is the theory that both cell types have the hemangioblast as the common ancestor. Moreover, cells derived from the hemangioblast, with either hematopoietic or endothelial differentiation capacity have been observed to share the expression of cluster of differentiation (CD) 34 and VEGFR-2 markers in adult humans, supporting the theory of the existence of an adult progenitor cell type in adults that can give origin to both hematopoietic and ECs.[8, 9] Later phenotypic characterization of the human hemangioblast itself determined the presence of VEGFR-2 (fetal liver kinase 1 in mouse), which has been studied in mouse embryos as essential for regulating both angiogenesis and vasculogenesis, as well as essential for the development of hematopoietic and ECs.[10] An important step in the cell lineage differentiation has been investigated through cell sorting, where progenitor cells with the VEGFR-2^{+} / vascular endothelial (VE)-cadherin^{+} phenotype determine the diverging point towards the EC lineage; on the contrary, VEGFR-2^{+} / VE-cadherin^{-} cells developed into hematopoietic cells (Figure 1).[11]

From the hemangioblast, a precursor cell termed the hemogenic endothelium differentiates into EPCs and hematopoietic precursors.[1] Interestingly, this step from hemogenic endothelium to the 2 types of progenitor cells does not occur by cell division, instead an endothelial to hematopoietic cell transition occurs.[12] This information provides evidence that EPCs share a common precursor with other lineages, making it possible to isolate them from different sources while maintaining a similar phenotype.

The different isolation methods of EPCs will be further discussed in a later section, nonetheless it is important to mention that two distinct EPC subtypes may emerge during the isolation process: 1) early EPCs, which are pro-angiogenic hematopoietic cells known also as circulating angiogenic cells, and 2) late outgrowth EPCs, also known as endothelial colony-forming cells (ECFCs).[7] This classification is based on the time of appearance of colonies in culture after isolation, their morphology, and expression of proteins.[12] For instance, early EPCs form colonies after 4-7 days in culture, and have predominantly spindle shape morphology, whereas late outgrowth EPC colonies appear at 2-4 weeks in culture and develop a cobblestone morphology, which is characteristic of ECs.[13] Furthermore, early EPCs will last no longer than 4 weeks in culture before gradually disappearing, as compared to up to 12 weeks for late EPCs.[14, 15]

As previously mentioned, surface proteins serve as markers to aid in the classification of EPCs and can be identified through immunophenotyping. Many cell surface markers such as CD34 are shared between early and late EPCs, whether others like CD31, VEGFR-2, VE-cadherin and von Willebrand factor (vWF), even when expressed strongly in late EPCs, can have weak expression in early EPCs. However, it has been observed that expression of VE-cadherin and

VEGFR-2 in early EPCs will decrease at 3 weeks in culture, while remaining strong in late EPCs. Even though strong expression of CD31 is characteristic of late EPCs, this surface protein is also expressed by mononuclear cells. As evidenced, different surface proteins are shared between early and late EPCs or even mononuclear cells, making phenotypic identification of a particular EPC challenging. Some other surface proteins such as CD45 and CD14 are more consistent, being expressed in early EPCs but absent in late ECFCs.[12, 15]

Endothelial progenitor cells do not refer to a single type of cell, but to at least 2 different cell populations with stem cell characteristics of self-renewability, clonogenicity and differentiation capacity.[12] Most importantly, and the property that makes them of interest for research in regenerative medicine, is their neovascularization capacities through either paracrine or autocrine mechanisms.[12] Early EPCs have pro-angiogenic capacity only through paracrine mechanisms, and are able to effectively release angiogenic factors such as VEGF and hepatocyte growth factor. Furthermore, early EPCs can elicit mitotic effect by releasing interleukin (IL) 8, enhancing EC survival and angiogenesis, and even stimulating EPC migration through secretion of stromal cell-derived factor-1 (SDF-1) and insulin-like growth factor-1 (IGF-1). Early EPCs have good paracrine function, while late EPCs tend to primarily secrete cytokines to maintain their main function, differentiate directly into ECs, and integrate into damaged vessels.[16] These characteristics make EPCs very good candidates to study regenerative angiogenesis for future therapeutic use, and although both early and late EPCs work together in EC repair, ECFCs have gained interested due to their intrinsic capacity of *de novo* vessel formation.

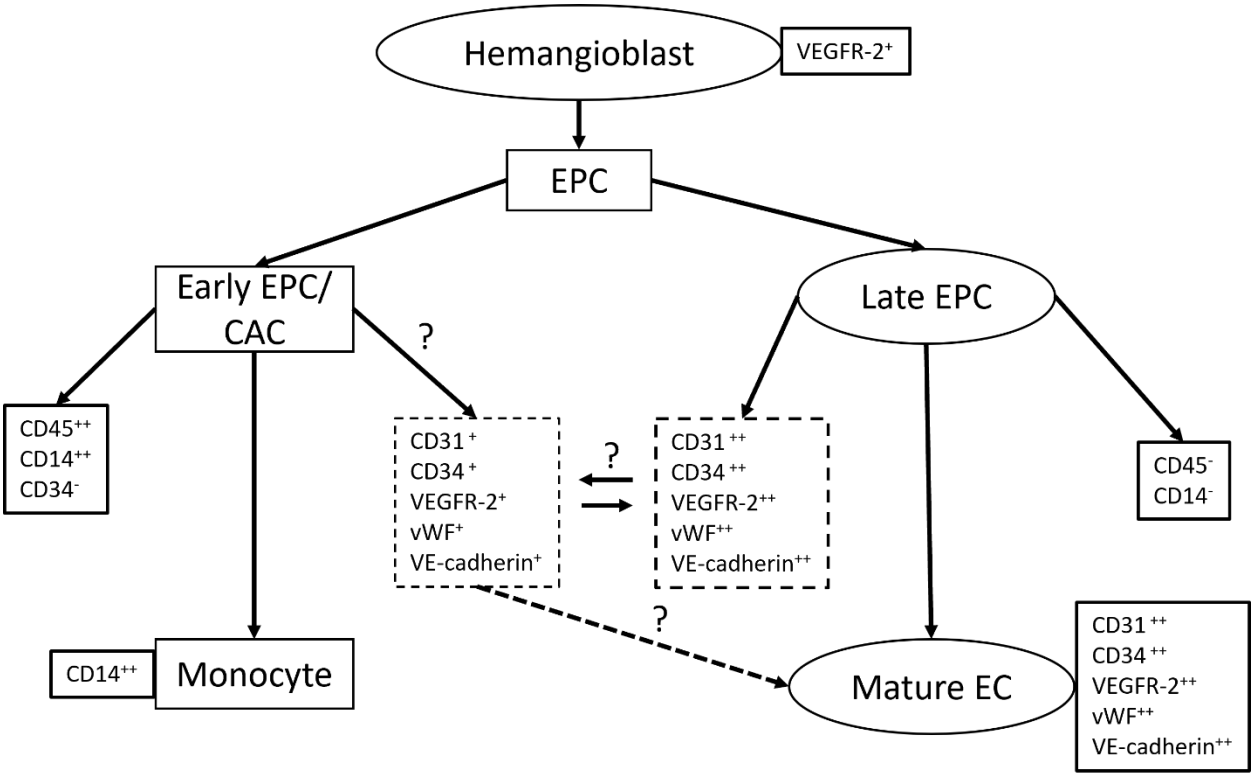


Figure 1. Endothelial progenitor cell origin. The hemangioblast gives origin to a common ancestor to the angiogenic and endothelial precursors. Further differentiation gives origin to early and late EPCs, which in turn will develop into monocytes or mature ECs respectively. However, some cell surface proteins, at different degrees of expression, are shared between them (dashed boxes), and a subset of monocyte precursor may also give origin to mature ECs (dashed arrow). CAC = circulating angiogenic cells; CD = cluster of differentiation; EC = endothelial cell; EPC = endothelial progenitor cell; VE = vascular endothelial; VEGFR-2 = vascular endothelial growth factor receptor-2; vWF = von Willebrand factor.

With the discovery of EPCs and more specifically the identification of late EPCs, known as ECFCs, it was determined that vasculogenesis was not limited to the embryonic development, but it was also possible in postnatal stages. These cells have the ability to differentiate into mature ECs and participate in *de novo* blood vessel formation as well as in endothelial repair at sites of vascular damage.[8, 9, 17] Endothelial colony-forming cells are truly endothelial precursors, which lack the hematopoietic or monocyte cell surface markers CD45, CD14, or CD115. These cells are able to uptake acetylated low-density lipoprotein (Ac-LDL), aggregate into bidimensional tube networks *in vitro* and originate patent vessels *in vivo*.[7, 18] There is no consensus in the phenotype of ECFCs for characterization, however they are known to express the cell surface markers CD31, CD105, CD144, CD146 and VEGFR-2, as well as having a positive expression of vWF.[18]

The origin of ECFCs is unclear, and it has been suggested that they arise from the bone marrow (BM), however this is inconsistent, and it is now believed they more likely originate from tissue vascular niches.[9] Endothelial colony-forming cells are known to reside within macrovessels, however they have also been isolated from microvessels of human placenta and white adipose tissue stromal vascular fraction. These microvascular ECFCs displayed better angiogenic properties compared to ECFCs derived from macrovessels.[7, 8] Following isolation, these cells can be amplified for multiple passages *in vitro* while maintaining differentiation potential.

The majority of ECFCs are quiescent, and their recruitment and mobilization from the niche into circulation is influenced by different cytokines and factors. Once released, they are recruited to sites of injury to enhance new blood vessel formation and vascular healing.[19]

Ischemia has been identified as one of the most important stimuli, and this has been observed in studies of peripheral ischemia as well as myocardial infarction. Hypoxia will increase the concentrations of VEGF in plasma, which is an important chemokine for ECFC mobilization.[5] The interaction between VEGF and its receptor will activate the enzyme nitric oxide synthase (NOS), producing nitric oxide (NO) and leading to the activation of matrix metalloproteinases. The activated matrix metalloproteinases will enhance and facilitate the mobilization of ECFCs into the peripheral circulation. One of the most important factors in ECFCs mobilization is SDF-1, which will be upregulated in the presence of inflammation and hypoxia. ECFCs highly express the SDF-1 receptor motif chemokine receptor 4, and upon their interaction, mobilization of ECFCs is initiated. It is also interesting that the mobilization mediated by SDF-1 is as well influenced by VEGF and endothelial NOS (eNOS). The formation of SDF-1 concentration gradients from the periphery to the ischemia site will trigger the migration of ECFCs.[19]

Endothelial colony-forming cells can stimulate vascular repair by different mechanisms: physical engraftment within emerging neovessels, paracrine release of pro-angiogenic mediators, secretion of microvesicles, and support of regenerative potential of mesenchymal stem cells (MSCs) and adipose stromal cells.[7] Even though they participate in pathways involving other cells, they also most likely differentiate into endothelium for direct repair.[8] Moreover, this new endothelium may have differences in functional properties and gene expression at different portions of the vascular tree, or organ specialization.[20, 21]

Endothelial colony-forming cells have the potential to produce angiogenic factors, self-replicate and differentiate into new blood vessels.[22] These are valuable characteristics when implementing therapies to increase blood supply in ischemic tissues, making ECFCs a potential

target therapy for ischemic conditions. Ultimately, in order to differentiate the putative EPC populations, it is necessary to evaluate their functional capabilities. An ECFC should be able to display clonogenic ability, form endothelial tubes with lumen *in vitro* when plated alone, and form *de novo* vessels *in vivo*.[18, 19, 23]

1.3 Characterization of endothelial colony-forming cells

1.3.1 Phenotypic characterization

As mentioned in previous sections, it is not fully clear where ECFCs originate from, but it is most likely that from multiple progenitor cell niches located within the vascular wall of blood vessels, including aorta, lung tissue, pulmonary artery, saphenous vein and placenta.[7] However, other sources such as white-adipose tissue or by manipulation in culture of human-induced pluripotent stem cells have proven to be successful in the isolation of ECFCs.[7] The main limitation regarding phenotypic characterization of ECFCs is the lack of a consensus on their molecular definition, and the difficulty in separating them from hematopoietic angiogenic cells.[23]. Nonetheless, ECFC phenotype in culture is frequently defined by the combination of expression of CD31, Ac-LDL uptake, and lectin binding.[23] However, since ECFCs express typical EC markers, including CD31, CD34, CD146, CD309, CD144, VEGFR-2, VE-cadherin, and vWF, researchers have used different combination of surface antigens to classify these cells, being the combination $CD34^+$ / $VEGFR\text{-}2^+$ one of the most commonly used.[8]. Moreover, to increase specificity during characterization of ECFCs, the lack of expression of hematopoietic cell

markers such as CD14, CD45 and CD115, as well as mesenchymal stem cell CD70 and α-smooth muscle actin antigens is often used in combination with EC markers.[7, 24]

In addition to the lack of agreement in the cell surface markers used for characterization, ECFC phenotype can be very dynamic, easily changing as a result of paracrine stimulation from other cell types, and differences in isolation and culture techniques between laboratories could as well be a source of inconsistency.[17] Additionally, other cell types like some monocyte subpopulations can express CD31 and VEGFR-2, making characterization less specific.[25] Another obstacle in defining ECFCs is the fact that Ac-LDL uptake is not specific to endothelial progenitors, and it can be also used to characterize both macrophages and mature ECs.[18] Other cell markers have also being used, including the stemness marker CD133, however some $CD133^+$ cells may result in hematopoietic cells.[23] Moreover, CD133 is present in the cytosol of ECFCs but not in the cell membrane, as observed with mature ECs, thus making it difficult to use as a surface protein.[9, 26]

Due to the previously mentioned difficulties in the phenotypic characterization of ECFCs, including the fact that the sole expression of surface proteins are not sufficient to define an ECFC *in vitro*, their morphology in culture, as well evaluation of angiogenic functions are well-accepted methods for characterization.[8] The timing of appearance of colonies as well as the morphology are important parameters prior to phenotypic characterization or function testing. Upon isolation, ECFCs will initially cluster having a polygonal and trigonal appearance before forming colonies after 10 days of culture. Colonies of late EPCs display a typical cobblestone EC morphology, and once the colony expands, specific flat radial cells often appear at the periphery.[27]

Phenotypic characterization of equine ECFCs has been recently reported from 2 studies that isolated circulating ECFCs from both jugular and cephalic veins of horses. Characterization was done based on expression of the cell markers vWF, VEGFR-2, CD34, and CD105 through indirect immunofluorescence. Additionally, equine ECFCs were also positive for CD105 and CD14 by flow cytometry; the other antibodies (vWF, VEGFR-2, CD34) were not successful for flow cytometry in those studies.[28, 29] The surface protein CD31 is highly conserved among several species, resulting in a broad species cross-reactivity, thus making it an option to be used in equine ECFCs.[4]

Although these markers are used to identify the EC phenotype, they do not determine the angiogenic capability, making the use of angiogenesis tests of utmost importance when evaluating these cells, both in culture and *in vivo*.

1.3.2 In vitro angiogenesis function

As previously mentioned, angiogenesis function tests are important to aid in the characterization of ECFCs, and *in vitro* tests are commonly performed as a first discriminator before testing is done in live subjects.

One of the most common function tests for ECFCs is the uptake of Ac-LDL, which is based on the principle that ECs will internalize Ac-LDL in a specific manner. Moreover, it has been correlated that cells that uptake Ac-LDL will have a typical endothelial phenotype of $CD31^+$ / $CD34^+$ / $CD45^-$.[30] In animal species, the uptake of Ac-LDL has been successfully used for the characterization of equine ECFCs *in vitro* in different studies, where about 60-75% were able to

uptake of Ac-LDL.[28, 29, 31] Although useful when used in conjunction with other function tests and immunophenotyping, the uptake of Ac-LDL lacks specificity for the characterization of ECFCs. Macrophages and mature ECs are able to internalize Ac-LDL, and it has been demonstrated that monocytes, both in culture and freshly sorted, underwent Ac-LDL uptake.[32]

A main characteristic of ECFCs is their ability to form tubular structures *in vitro* when cultured alone in a basement membrane, therefore this is one of the most common assays performed to evaluate ECFC function.[22] Matrigel®, a type of basement membrane derived from Engelbreth-Holm-Swarm mouse sarcoma, which is a tumor rich in extracellular matrix proteins, is the most common type of matrix used for *in vitro* tubulogenesis assays. This basement membrane has both structural and biological functions, and although not exactly the same composition as EC basement membrane *in vivo*, it contains laminin-1, collagen IV, heparan sulfate proteoglycan, and various growth factors. This composition resembles the *in vivo* extracellular matrix layer in contact with the basal surface of the ECs, which helps maintain the tube-like structure of the blood vessels. *In vivo*, this process of tubulogenesis has the function of connecting existing vasculature to new blood vessels by migrating and reassembling into the extracellular matrix. It is believed that the formation of capillary-like structures is specific of ECs, thus the fact that early EPCs and ECFCs are able to form tubules *in vitro* is an example of cell differentiation on basement membrane that recapitulates the many steps of angiogenesis.[33] Even though this assay is widely used to evaluate the vasculogenesis capacity of ECFCs, other cells such as fibroblasts and monocytes cultured under endothelial conditions can also form vessel-like structures, and making a distinction between them is difficult with the

use of light microscopy alone.[34] Evaluation of a cross section of the extracellular matrix containing the microtubule may revealed the presence of a lumen, and further immunostaining can aid in differentiating ECs from other cell types.[35]

Angiogenesis is a very complex process, and the *in vitro* tubule formation assay is able to replicate many of the steps of angiogenesis, including adhesion, migration, protease activity, alignment and tube formation.[33] This is a very versatile first screen assay to study compounds with potential pro- or anti-angiogenic properties, often times followed by *ex vivo* or *in vivo* tests.[34, 36] However it is an assay that can have many variables, and results will vary depending on the cell seeding density, volume of Matrigel® used, as well as the cell passage number.[33] Although this test is widely used, the interpretation of results varies among researchers and laboratories. Since there is no common method used to quantify vessel-like structures, there are variations in the analysis, making it difficult to compare between different studies.[37] Different methods have been described to quantify tubules using this assay, commonly starting by the acquisition of the image and modifications using different software (ImageJ, Adobe® Photoshop). Manual counting of tubules has been commonly done, however this lacks objectivity and is generally more time consuming.[37]

With the availability of computer software, it is possible to more objectively quantify tubules. For instance, the angiogenesis analyzer, a plugin from ImageJ, is able to quantify different parameters of tubule formation, including the number of branches, tubules, junctions, nodes, meshes, tube length and mesh area. This tool has been used in Matrigel® tubule formation from equine ECFCs, where the effect of recombinant IL-1β in *in vitro* vasculogenesis was studied. In this case, ImageJ processing and the angiogenesis analyzer proved to be useful

in quantifying the number of segments, branches, meshes, junctions, tube length and total mesh area.[38] Studies of tubule formation in Matrigel® using equine ECFCs have also used the presence or absence of tubules alone[29], or have developed a scoring system from 1-4 where 1 corresponds to no evidence of tubule formation, 2 where projecting tubes are observed but with no connection, 3 vascular tubule formation is evident with connecting tubules in ≤50% of the field, and 4 if connecting tubules are evident in >50% of the field.[28, 31]

The Matrigel® tubule formation assay, although useful as the first method in function studies with ECFCs lacks the different molecular and cell interactions seen *in vivo*, and ideally for an assay to be translatable, it should evaluate the formation of functional blood vessels, requiring the interplay with perivascular supporting cells, as well as the processing of extracellular matrix in order to expand and form networks.[22]

1.3.3 Ex vivo angiogenesis function - The arterial ring assay

Endothelial colony-forming cells have the important function of stimulating blood vessel formation and repair, making them good candidates to study cell-based therapy in both human and animals.[39] However, the lack of consensus in their phenotypic definition makes *in vitro* studies difficult to interpret.[40] Since angiogenesis involves many cellular and molecular processes, *in vivo* studies are warranted, but this comes with the limitations of being more expensive, invasive, often times difficult to analyze, and may raise ethical concerns.[39]

Ex vivo models of angiogenesis using vascular explant cultures are useful in evaluating angiogenesis because they overcome the limitations of *in vitro* techniques, while reducing the

complexity of *in vivo* models, thus bridging the gap between these techniques.[41, 42] E*x vivo* models are able to mimic most of the steps of angiogenesis seen *in vivo*, such as initial sprouting, matrix remodeling and lumen formation. Paracrine signaling between endothelial cells, fibroblasts, macrophages and pericytes are critical in formation of vascular tubes, which can be mimicked in *ex vivo* assays.[42, 43]

The mouse aortic ring assay is a common *ex vivo* model to study vascular formation, being suitable in both anti- and pro-angiogenic studies. Aortic explants have the capacity to form branching microvessels when embedded in extracellular matrix. Furthermore, it is relatively low cost with the benefit of no interventions performed in live subjects.[44] Although there is inherent variability in the angiogenic response of rings from the same animal, this system can be adapted to different experimental conditions, and the endothelium of the explants behaves similarly to EC *in vivo*.[43, 45] This assay has been used in other species such as chick embryos, dogs, humans, cows, and a single report of the use in horses.[39, 42] Equine arterial rings were used to evaluate the effects of cortisol on angiogenesis, by adapting the mouse aortic ring to equine facial and laminar arteries.[46]

This test, originally developed in rats and mice, consists of the culture of aortic segments usually measuring about 1 mm in diameter, which are embedded in a basement membrane, typically collagen or Matrigel®. These rings are cultured in endothelial growth media (EGM) and can be exposed to different agents to study the effects on angiogenesis.[34] The aortic ring will produce tubular structures originating from the walls of the tissue explant, and are often easy to observe and quantify; moreover, due to the nature of the tissue used, vascular supportive cells will be available. This *ex vivo* test is typically used to study the

angiogenic effects of secretory proteins, drugs, or responses in transgenic mouse models. Moreover, it has been described as a powerful tool to evaluate the angiogenic effect of cells in regenerative therapy. The co-culture of human MSCs in the mouse aortic ring model has shown incorporation of these cells into the vascular structures, enhancing the radius and loops of the vascular network.[22, 47]

1.3.4 In vivo angiogenesis function

In vivo assays should be used as a final confirmation of the results of *in vitro* or *ex vivo* analyses. A common *in vivo* test is the Matrigel® plug assay, where the basement membrane is injected subcutaneously in the animal, commonly a mouse. When liquid at 4°C, Matrigel® can be mixed in a suspension with the compound of interest, which can be cells or other angiogenic factors, and once solidified at body temperature, it will allow for incorporation of new blood vessels from the host.[34, 48] The Matrigel® plug can be excised and analyzed within a week by techniques such as immunofluorescence or histology for evaluation of angiogenesis.[34, 44] Commonly, immunological staining for CD31 and/or CC34 is used to assess neovascularization growth into the plug.[48] In a study evaluating the angiogenic effects of human monocytes cultured under EC conditions, the Matrigel® containing the study cells was injected into mice to form a plug, which resulted in more exuberant vasculature when compared to the use of Matrigel® alone.[49]

To date, there are no reports of using this assay with equine ECFCs or using the horse as the host. This assay has the limitations that, at least in mice, the age and gender can influence

the results. Furthermore, the Matrigel® itself can lead to inconsistent results depending on the degree of dilution from the added substance, or the formation of excessive bubbles, both factors that can affect the structural properties of the gel.[44]

Other *in vivo* murine angiogenesis models study the development of collateral circulation and changes in blood flow following ischemia of the hind limb.[50] Moreover, different methods of unilateral hind limb ischemia have been described, from simple ligation of the femoral or iliac arteries, to a complete excision of the artery for a more severe ischemia. The protocol for hind limb ischemia chosen will largely depend on the goals of the study.[51] For analysis of blood flow recovery, laser doppler imaging is traditionally used, and sometimes this can be combined with functional test to evaluate the motion of the limb. In order to evaluate neovascularization, immunohistochemical analyses are commonly used, for example using EC staining for CD31 and vWF.[44]

The angiogenic capacity of human ECFCs have been extensively evaluated using the hind limb ischemia model. Following ligation of the femoral artery in rats, it was observed that intramuscular and intravenous injection of human ECFCs increased significantly the blood perfusion in the ischemic limb, and immunohistochemical staining showed greater number of CD31-positive cells in the ischemic muscles.[52] However, there is variability in the response to this assay in mice due to the differences in neovascularization capacity among different individuals. Similarly, this is commonly performed in healthy young animals, which will not truly represent the population affected by poor neovascularization due to comorbidities.[44]

Angiogenic function tests are widely accepted for characterization of ECFCs due to the limitations of phenotypic characterization alone; however, some of the current isolation and culture protocols face difficulties in avoiding the overgrowth of other cell types and the maintenance of their phenotype when propagated.

1.4 Isolation of endothelial colony-forming cells

Different factors will play a role into the successful isolation and expansion of ECFCs, where the type of substrate used for cell attachment as well as the EGM and media supplements will favor the selection of cells with an EC phenotype. In order to overcome some of the challenges in ECFC isolation and propagation, techniques such as cell sorting based on the expression of EC markers, including CD31, and the addition of alternative sources of vascular growth factors can be implemented.

One of the challenges in the isolation of ECFCs is the low number in circulation, which has been estimated to be about 0.06 cells/mL of whole blood and 0.05% of the mononuclear cell fraction.[53] Moreover, there is no consensus on the protocol for isolation, and contamination with other cell types is very common.[12] Since these cells share common precursors with other lineages, they can be isolated from different sources such as BM, however isolation is very challenging due to the heterogenous mixture of hematogenous and MSCs residing in close proximity. Therefore, and based on the principle that peripheral blood cells originate from BM, there has been a shift towards isolation of ECFCs from circulation, which is also a less invasive procedure as compared to BM harvest. Another commonly used

source of whole blood for isolation of ECFCs is umbilical cord blood. In humans, some reports found that isolation of ECFCs from umbilical cord blood resulted in greater number and larger size of colonies when compared to peripheral blood.[54] It is also known that ECFCs could arise from progenitor cell niches located within the vascular wall of large vessels, veins, placenta or adipose tissue.[7] This fact makes it possible to isolate ECFCs from different tissue sources, such has been done in human umbilical cord and adipose tissue.[12] However, differences have been observed in phenotype and function of ECFCs depending on the source. For instance, human ECFCs isolated from term placentas with the $CD31^+$ / $CD144^+$ / $CD105^+$ / $VEGFR2^+$ / $CD34^-$ / $CD45^-$ phenotype demonstrated greater *in vivo* vasculogenic potential than circulating ECFCs from umbilical cord blood.[55]

When it comes to isolation of ECFCs, there are not differences just in the source, but in the isolation protocols as well. Different isolation techniques have been successfully used to isolate ECFCs from either whole blood or BM. By using density gradient centrifugation (DGC), the mononuclear cell fraction is plated on a rat tail type 1 collagen-coated dish, and around day 4 of culture the non-adherent cells are removed. The 4-day mark has been used because the unwanted platelets, red blood cells, or monocytes are gradually depleted over this period, however this number of days is not fixed and often modified by researchers.[12] ECFC colonies displaying a cobblestone morphology, which is typical of ECs in culture, usually appear from the adherent cell population after 10-21 days of culture.[7] Another isolation method requires the use of whole blood in supplemented EGM, however by this method there is more contamination with platelets.[19]

Isolation of a pure population of ECFCs is difficult, therefore, modifications in protocols, as well as the use of a combination of different techniques have been described. When evaluating the initial cell clusters and colony formation, cells or colonies that do not display a cobblestone morphology can be removed with a cell scrapper before they infiltrate into ECFCs colonies.[27] Likewise, colonies with a homogeneous cobblestone morphology can be isolated using cloning cylinders before being dissociated for replating and further expansion.[56] These techniques are useful to avoid growth of undesired cells, and can be further refined by combining with immunophenotyping selection, therefore preserving the desired phenotype.

1.4.1 Cell sorting for ECFC isolation

Further refinement in cell isolation protocols uses the aid of molecular techniques, specifically cell sorting. This technique has the potential to enrich or purify cell samples into well-defined populations to enhance the efficiency in research or clinical applications. The goal is for the isolation of rarer cell populations, which is the case of circulating ECFCs.[57]

Cell sorting can be done by 2 different techniques, either magnetic activated cell sorting (MACS) or fluorescence activated cell sorting (FACS). In the first method, the cells are labelled with the antibody of interest, and this antibody attached to magnetic beads based on the same isotype. The labeled cells are then attached to magnets and the unwanted cells will flow as the eluted fraction. This system allows for faster sorting rates, requires less specialized equipment, and uses less diluent volumes when compared to FACS.[57] Furthermore, since it is a faster method when compared to FACS, multiple rounds of sorting can be done to improve the purity

percentage. In the isolation of ECFCs from rat peripheral blood based on expression of CD36, the first cell sorting resulted in a purity of 76%, and a second round of MACS increased this to a 90%.[58] However, MACS only sorts based on presence or absence of the antigen, but lacks the ability to grade according to the expression level.[59]

In the case of FACS, it relies on fluorescent probes to identify cells by type. Cells within a liquid stream passing through the laser light and optics are split into droplets that are charged and electrostatically deflected into the different groups.[57, 59] Moreover, FACS allows for both single cell selection as well as a quantification of expression.[59]

Due to the low number of circulating ECFCs, thus being a rare cell population, cell sorting techniques have been used for isolation based on the expression of EC cell surface markers, such as CD144, CD31 or VEGFR-2 among others.[55, 60] This selective isolation can be done directly from whole blood, BM or the peripheral blood mononuclear cells (PBMC) fraction following DGC.[12] Cell sorting done directly from peripheral blood samples have the advantage of using smaller blood volumes, which will be of benefit when isolating ECFCs from small species, however the number of cells obtained will be small. The other method of using cell sorting for the isolation of ECFC consist in isolating and expanding the ECFCs *in vitro* from peripheral blood as previously described, followed by cell sorting and expansion of the cells of interest. Since this method results in a larger cell yield, multiple cell surface markers can be used simultaneously or by consecutive sortings, thus allowing for a more detailed cell characterization.[61]

In the isolation of human ECFCs, a method that has proven to be effective is the use of MACS from peripheral whole blood based on expression of CD31, followed by plating of the $CD31^+$ cell fraction and further expansion of the cell colonies.[62] Regarding the use of cell sorting in animal models, most of the reports correspond to murine models. Mouse ECFCs have been obtained following MACS from buffy coat following DGC based on expression of CD31, CD36 and CD34.[58] Similarly, the use of FACS has also been successful in isolating circulating cells from rat whole blood that were positive for the uptake of Ac-LDL, but at the same time negative for the myeloid marker CD11b/c. This method allowed for a positive and negative gating selection, where also cells from monocytic/granulocytic origin that can take Ac-LDL were excluded.[58]

In large animals, MACS has been used for positive selection of granulosa cells based on expression of CD14 and CD45. Subculture of this somatic progenitor cells gave origin to ECs that were positive for VEGFR-2. Although cell sorting for isolation of rare cell populations has been used in large animals, there are no reports of its use for isolation of ECFCs from peripheral blood.

1.4.2 ECFC isolation in horses

Isolation of ECFCs has been previously described in horses from peripheral blood obtained from either the jugular or cephalic vein.[29] Initially, isolation from jugular vein whole blood was successful in forming colonies in 3 out of 24 horses, and the time of appearance around 12 days was consistent with late EPCs. Moreover, these equine cells were positive for

the EC markers vWF, VEGFR-2, CD34 and CD105 by indirect immunofluorescence, however they also expressed the surface protein CD14, which is characteristic of myeloid cells.[28] Further investigation in the isolation of equine ECFCs described the use of DGC for plating of the PBMCs fraction from whole blood. Moreover, greater success in isolation of equine ECFCs was achieved when using vessels of lesser diameter than the jugular, in this case the cephalic vein, where more colonies with cobblestone morphology were retrieved when compared to samples from jugular vein or whole blood isolation.[29] Functionally, equine ECFCs previously isolated from these studies were able to uptake Ac-LDL and form tubules when cultured *in vitro* in basement membrane, both desired characteristic of ECFCs.[28, 29]

Even though efforts have improved the isolation and culture of ECFCs, both in humans and horses, the lack of consensus in their phenotypic definition and the very low number of cells in circulation, makes it challenging to obtain a pure population after expansion in culture. Culture conditions for the isolation and expansion of ECFCs play an important role in defining the phenotypic fate of these cells. For isolation and cell expansion, coating the culture surface with extracellular matrix protein will promote adhesion and growth, however the type of matrix used could favor the adhesion of certain types of cells. For instance, ECFCs cultured in collagen-coated plates achieved a higher number of passages and longer lifespan when compared to fibronectin-coated plates.[61] However, with the attachment of EPCs during isolation there is also the presence of platelets, that can co-fractionate with the PBMCs. These contaminating platelets will attach to the adherent PBMCs, and platelet membrane proteins can be transferred to the adherent cells, including the platelet-derived surface antigen CD31, thus mistakenly identifying these mononuclear cells as ECFCs.[18] Most importantly, it is well known

that the type of cell culture media, and the growth factors contained within will influence the cell phenotype. For instance, even human monocytes, when cultured under ECs conditions and stimulated with VEGF, resulted in an increase expression of CD31 while expression of CD14 decreased.[49]

1.5 Cell expansion of endothelial colony-forming cells

The selection of an appropriate cell culture media will have an impact on the research results and further clinical applicability. The cell culture media has a very important function in supporting cell survival, proliferation, and cellular functions, therefore depending on the aims or results of the study, the researcher might have to modify the composition of the growth media. The cell growth media is composed of basal media, the added growth factors, and (frequently) serum, and this composition is tailored towards the cell type, origin animal, and the purpose of the culturing. If using serum, basal media will be composed of just the minimum necessary components such as inorganic salts, sugar, essential amino acids and water-soluble vitamins.

Serum can be obtained from different animal species, and when added to the cell growth media, it provides a variety of active substances necessary for the survival and growth of the animal cells. Even though most of commercially available cell growth medias contain growth factors including epidermal growth factor, insulin-like growth factor, fibroblast growth factor, and transforming growth factor, the effect in cell proliferation will almost always be inferior to the use of serum[63] This could be in part due to the fact that insulin acts in a

coordinated manner with serum components to stimulate cell growth.[64] Fetal bovine serum (FBS) is the most popular and widely used serum, and in contrast to calf serum, it is richer in growth factors and has low levels of γ-globulins, which has a cell growth inhibitory activity. Fetal bovine serum has been commonly used for the culture of ECs and ECFCs in humans, however its xenogeneic origin increases the risks for xeno-immunization against bovine antigens, transmission of pathogens due to microbial contamination, as well as ethical concerns when it comes to the harvesting process. [65] Furthermore, FBS has the disadvantage of having batch-to-batch variability, resulting in unpredictable concentrations of its biological components which can lead to culture results that are less reproducible.[63, 66] On the other hand, horse serum (HS) obtained from adult horses has shown to be more homogenous between batches, and has the advantage of being of allogenic origin when culturing equine-origin cells.[63] Different studies have successfully used HS for the isolation and expansion of equine ECFCs, which have resulted in adequate cell proliferation.[28, 29] However, there are no studies comparing the effects in phenotype or function of equine ECFCs between culture with FBS and HS.

1.5.1 Equine platelet lysate as an alternative source of growth factors

Further improvement of ECFC culture conditions may include the use of alternative sources of growth factors, and different studies have focused on the effect of human platelet lysate in culture of ECFCs. Within the α granules, platelets store high concentrations of growth factors, including VEGF-A, IGF-1, transforming growth factor-β (TGF-β), and SDF-1, which are

tightly related to vascular growth and angiogenesis. Moreover, platelets can be artificially activated to release the factors and use them as enhancers in cell culture.[65]

Studies of cultured human ECFCs have shown that platelet lysate improves cell survival, vasculogenic activity, and yielded more colonies during isolation.[67-69] This increase in angiogenic activity has been demonstrated both *in vitro* and *in vivo* in mouse ischemic models.[67] Even though different concentrations of platelet lysate has been used in humans, the use of 10% supplementation in the EGM, resulted in ECFCs with genetic stability for more than 30 population doublings.[70] Moreover, platelet lysate has also proven to be effective as a bio-scaffold for 3D ECFC culture, where co-culture within a platelet lysate gel resulted in greater number of vascular structures with increased expression of CD31.[67]

The successful use of platelet lysate as a source of growth factors in the culture of human-origin cells was the starting point towards its use in animal species. Specifically, equine platelet lysate (ePL) has been used for the propagation of equine MSCs, and when added at 10% to the growth media, was observed to perform similarly to the control FBS regarding cell phenotype expression, proliferation, and trilineage differentiation.[71, 72] Furthermore, MSCs cultured with ePL had superior cell viability after a washing step when compared to FBS.[72] Interestingly, MSC proliferation has been observed to be inconsistent or absent when adding the ePL at 2.5% or 5% concentration instead of the commonly used 10%.[73] Although the concentration of ePL in the cell growth media has shown to have an effect on equine MSCs, other factors such as the method used for platelet activation and variability among animals can influence the concentration of growth factors in the lysate.

Platelet lysate can be generated from platelet-rich plasma by different methods, resulting in different composition of growth factors and cytokines. Two different methods are commonly used in veterinary medicine, freeze-thawing to physically disrupt platelet membranes or adding calcium and autologous serum to induce physiological activation of platelets.[74] Textor *et al*.,[75] found that concentrations of the growth factor platelet-derived growth factor-BB (PDGF-BB) was higher after calcium chloride activation when compared to a single cycle of freeze-thaw, however, a different study reported that the use of multiple freeze-thaw cycles resulted in greater concentrations of PDGF-BB and TGF-β1 in ePL.[74, 75]

Additionally, variability in the ePL growth factor concentrations among horses has been documented, and it can vary according to intrinsic variables such as breed, gender and age, where younger and female subjects resulted in higher concentrations of PDGF-BB.[76] Although horse variability has been reported to be small for concentrations of PDGF-BB and TGF-β1 in ePL from horses, large ranges were observed in VEGF-A concentrations. [77] Due to this variability, the use of pooled ePL is recommended, which has demonstrated to result in enhanced chondrogenesis when used for equine MSCs in culture.[72]. In the contrary, using autologous ePL resulted in no significant changes on chondrogenic or osteogenic differentiation.[78] However, the use of autologous ePL has the advantage of reducing the risk of transmission through plasma-borne viruses, however the production of larger volumes of large-scale cell expansion might be a significant limitation.[78]

Culture of human ECFCs has been traditionally done by supplementing the EGM with FBS, however this raises concerns for immunogenic reactions due to its xenogenic origin. For instance, the presence of retained animal proteins within the cytoplasm of cultured MSCs has

been reported in both humans and horses, and FBS-cultured MSCs have been recognized for cell death by recipient horses after intra-articular injection.[79, 80] In addition to the xenogenic potential of the use of FBS, its production also raises ethical concerns, therefore the use of allogenic, easy-to-access sources of growth factors is desirable.

When culturing ECFCs of equine origin, horse blood-derived components such as HS have been successfully used as a supplement of EGM for cell expansion after isolation.[29] However, concentration of growth factors such as PDGF-BB from pooled ePL is higher than in HS.[77] Therefore, the use of biologicals of allogenic origin and with higher concentrations of growth factors, such as the ones contained in ePL, may enhance the equine specific angiogenic pathways.

1.5.2 Nitric oxide donors for cell culture

Physiological laminar blood shear forces are in direct contact with ECs and will stimulate signaling pathways for the initiation of cellular responses. Endothelial cells are able to sense these shear forces, mainly through integrins, VEGFR-2, ion channels, G-protein coupled receptors and platelet endothelial cell adhesion molecule-1. Moreover, this activation of the VEGFR-2, promotes the downstream activity of the phosphoinositide 3-kinase/ protein kinase B (PI3K/Akt) pathway, stimulating eNOS phosphorylation and NO production (Figure 2).[81] Signaling via receptors, such as VEGFR-2, as well as interactions with other proteins, regulate eNOS activity and NO production.[82] It has also been observed that laminar shear stress, through the NO activation pathway will have an anti-apoptotic effect and promote cell

proliferation of ECs.[81] Moreover, NO plays a role in the different key processes of angiogenesis, including dissolution of matrix, EC proliferation and migration and the final formation of tubules.[83] However, static shear forces, or changes in flow pattern, either in direction or magnitude, can decrease the activity of eNOS, leading to prothrombic and proinflammatory states.[84] These effects were observed when challenging human umbilical vein ECs (HUVEC) to different shear stress magnitudes, where the expression of CD31 was significantly decreased in cells under static culture when compared to dynamic.[84]

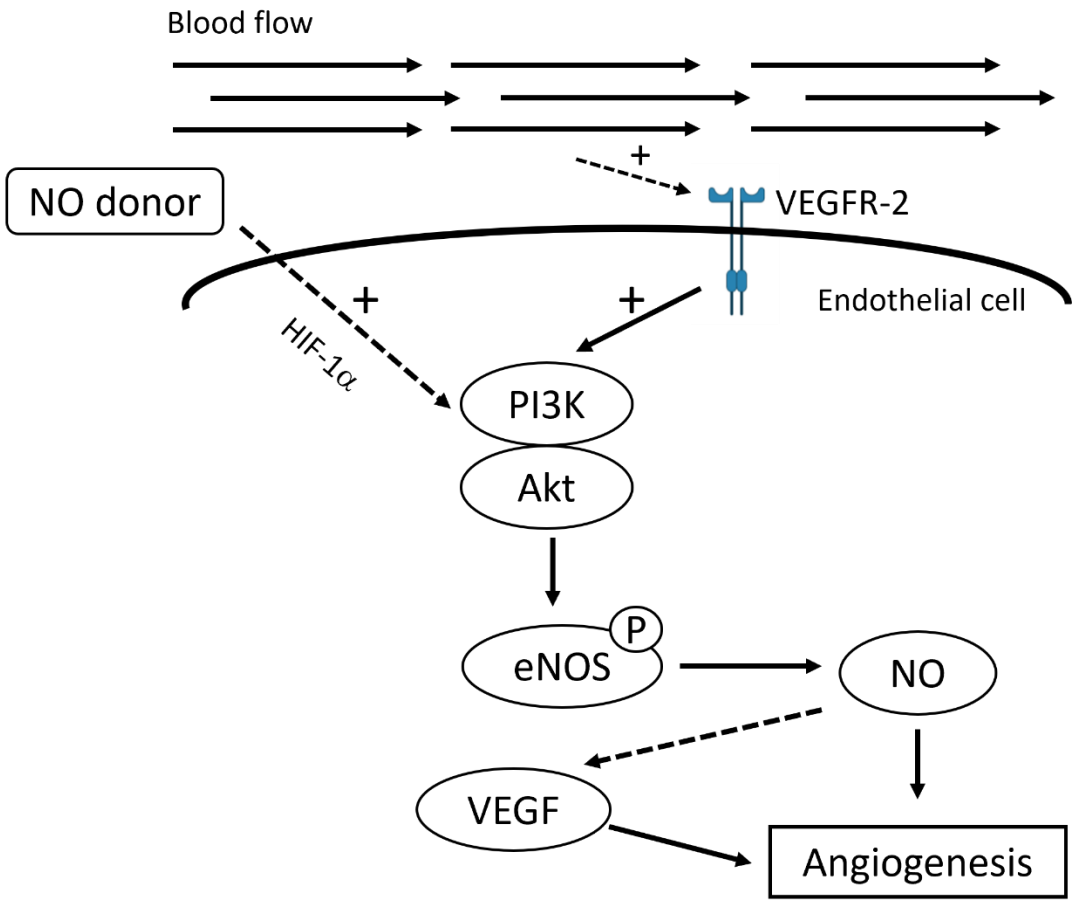


Figure 2. Effect of shear stress on nitric oxide production. Endothelial cells *in vivo* are in contact with physiological laminar shear forces that will be sensed by receptors such as VEGFR-2. The activation of this receptor will stimulate the PI3K/Akt pathway downstream to phosphorylate eNOS. This will increase the release of NO, important for angiogenesis. At the same time, NO will also increase VEGF synthesis, and this can be augmented by the addition of a NO donor, which is able to stimulate the same pathway through the HIF-1α present in the cytoplasm (dashed arrow). Akt = protein kinase B; eNOS = endothelial nitric oxide synthase; HIF-1α = hypoxia-inducible factor 1-α; NO = nitric oxide; PI3K = phosphoinositide 3-kinase; VEGF = vascular endothelial growth factor; VEGFR-2 = vascular endothelial growth factor receptor-2.

Although the effects of laminar shear stress are desirable, this sometimes is difficult to reproduce in cultured cell. Dynamic cultures often require the use of specialized equipment that can be costly or difficult to access. Physiologic shear forces have been simulated in cell cultures by using an orbital shaker rotated at 210 cycles/min to produce 12 dynes at the periphery of the wells, which is close to the average shear in the human femoral artery. This method in the culture of human adipose stem cells resulted in a shift towards an EC phenotype, with an increase in the expression of CD31 and uptake of Ac-LDL.[85] Although dynamic cultures are ideal to stimulate the different receptors and pathways implicated in EC function, the addition of exogenous NO is a way to overcome the difficulties of dynamic cell culture.

Nitric oxide is an endothelium-derived relaxing factor with functions of vasodilation, vascular permeability, and antithrombosis. Furthermore, it has cardiovascular homeostasis and vasoprotective properties, and it may play an important role in cardiovascular regenerative processes, primarily by promoting cell proliferation.[86, 87] Nitric oxide results from the conversion of L-arginine to L-citrulline, which is mediated by the endothelial isoform of NOS, eNOS.[19, 86] Nitric oxide, being a gaseous radical, is continuously synthesized within ECs due to its short half-life, and acts as a chemical messenger primarily in the vascular and immune systems.[88] Furthermore, eNOS is highly expressed in vascular ECs as well as in EPCs, and likewise, VEGFR-2 will be the receptor initiating the cascade of events leading to the production of NO for EPC mobilization and function.[19, 87] Although it is well known that VEGF-A upregulates the expression of eNOS, leading to the release of NO, this cascade of events may also occur in the opposite direction, with NO upregulating the production of VEGF-A. In rat and human vascular smooth muscle cells, the release of VEGF into the culture media, as well as the

VEGF mRNA expression was significantly higher in the presence of NO donors 3-morpholinosydnonimine and S-nitroso-N-acetylpenicillamine (SNAP). Furthermore, this upregulation was reversed by the NOS inhibitor L-N^G-Nitro arginine methyl ester (L-NAME).[83] Similar results have been observed with increasing protein expression of VEGF following SNAP treatment of cultured rat cardiomyocytes. Additionally, in cultured HUVECS, greater *in vitro* angiogenesis was observed when a NO donor was added to the EGM, and interestingly this NO donor activated the angiogenic signaling pathway PI3K/Akt through hypoxia-inducible factor-1α (HIF-1α), which is one of the most powerful factors to stimulate angiogenesis.[89]

The effects of NO in angiogenesis has been also studied in *in vivo* models, where eNOS-depleted mice had a significant reduction in EPC function, and EPCs pretreated with an eNOS enhancer improved neovascularization in a limb ischemia model.[87] Nitric oxide has the benefit of being a potent signaling molecule and cytoprotective at low concentrations; however, it will have a cytotoxic effect at high concentrations.[90, 91] The cytotoxicity of NO is mostly due to its involvement in the synthesis of peroxynitrite, produced from the interaction between NO and the free radical superoxide anion. Ultimately, peroxynitrite interacts with lipids, DNA, and proteins triggering changes in cell signaling or oxidative injury.[91] In physiological conditions, concentrations of NO as low as 10 nM are produced by eNOS, acting both as vasodilator and inhibitor of platelet aggregation; however during inflammation, excessive amounts of NO (above 1 mM) can be produced, especially by the inducible NOS isoform, which modulates the activity of inflammatory mediators.[88] The various effects of NO will depend on the site of synthesis, concentrations released, and the type of target tissue.

The use of NO donors is gaining interest to improve EC and ECFC cell culture conditions, and as a therapeutic option in the treatment of cardiovascular diseases. The different NO donors can release NO by different mechanisms, either spontaneously, by enzymatic reaction, or chemical catalysis. Spontaneous NO donation will release NO by thermal or photochemical self-decomposition, while some others will depend on enzymatic oxidation or reduction. When the NO donor reacts with acids, alkali, metals or thiols, it will trigger a reaction to release NO. This is the case of the NO donor S-nitroso cysteine (CysNO), a NO donor from the S-nitrosothiols class, which has been commonly used in culture of different cell types.[88]

The effect of CysNO has been evaluated in cardiomyocyte differentiation of both mouse and human cell suspensions, providing a more inexpensive method for differentiation in a large-scale cell culture.[92, 93] Furthermore, the effects of NO on other cell types such as EC and EPCs, showed to be beneficial. *In vivo*, when a NO donor was delivered by a NO-containing hydrogel following carotid balloon injury in mice, an increase in EC proliferation was achieved.[94] Moreover, the addition a NO donor to L-NAME treated or eNOS deficient mice resulted in an increase in BM EPC mobilization, and this seemed to favor particularly EPCs since no effect on recruitment of hematopoietic stem cells was observed.[95] Although there are no reports of the use of NO donors in the culture of equine ECs or ECFCs, the effects of NO in cultured EPCs have been studied in human cells, and one study found an improvement in cell function based on enhanced tube formation *in vitro*.[13, 19] Similarly, when human EPCs were subjected to hypoxia in culture, the addition of a NO donor promoted cell-matrix adhesion, thus preventing the deleterious structural effects of hypoxia.[96]

Large scale culture of ECFCs is gaining popularity to be used in regenerative angiogenesis, and improving culture methods such as the use of dynamic cell culture or the addition of NO is important to stimulate the angiogenic pathway and preserve the phenotype for future therapeutic use.

1.6 Regenerative angiogenesis

1.6.1 Human regenerative medicine

A wider therapeutic use of ECFCs has been prevented by the inconsistency in definitions, characterization, and different protocols for both isolation and expansion from peripheral blood or umbilical cord.[7] It has become a priority in human medicine to improve the methodology for ECFC isolation and expansion *in vitro,* and efforts have been made to improve culture conditions, such as the replacement of FBS with human platelet lysate in the culture media.[23]

In humans, ischemic heart disease is one of the main contributors of cardiovascular disease, and the origin of this is usually endothelial dysfunction.[97] Often, surgical intervention is not feasible to restore blood flow, and pharmacological treatment is necessary. Therefore, alternative therapeutic approaches to promote neovascularization in ischemic tissues is desirable.[7] The goal of therapeutic angiogenesis is to promote angiogenesis and vasculogenesis, and this can be achieved by the administration of pro-angiogenic genes or peptides, as well as using cell-based therapies.[7] The latter consists of the mobilization or transplantation of pro-angiogenic stem cells, including MSCs, hematopoietic cells and EPCs.[7]

As previously mentioned, in contrast to early EPCs, ECFCs have more angiogenic potential due to their capacity to differentiate into mature ECs and form new functional blood vessels in areas of ischemia.

Endothelial colony-forming cells of human origin transplanted to mice or rats have shown to contribute to vascular regeneration in ischemic tissues such as heart, brain, retina and limbs.[8] ECFC transplantation proved to effectively treat ischemic brain injury in a mouse model by improving angiogenesis, decreasing neuronal apoptosis, and enhancing neurogenesis while increasing the release of pro-angiogenic substances such as VEGF.[98, 99] Moreover, human ECFCs have been tracked post-implantation and observed to assemble into functional vessels that integrate with the host vasculature, staying functional for at least 3 months following injection in a rat ischemia model.[100] Therapeutic angiogenesis has been extensively investigated for the treatment of lower limb ischemia in humans, which often leads to amputation or death. To study this, murine models are most commonly used, and it has been observed that ECFCs infused intravenously rapidly relocate to the ischemic limb and restore the blood perfusion, further showing that ECFCs promote neovascularization by direct incorporation within the neovessels as well as by paracrine stimulation.[101]

The efficacy of ECFCs in restoration of angiogenesis could be enhanced if combined with other cell types such as MSCs or myeloid angiogenic cells, which will provide paracrine signaling to improve the function of ECFCs.[102, 103] However, in order to reach the clinical use of ECFCs in humans and animals, different strategies should be considered in order to properly expand them in culture, prolong their viability during delivery, and stimulate them via paracrine mechanisms to enhance the vasoreactive activity.[7] Furthermore, in humans, ECFCs from

individuals experiencing cardiovascular disease formed colonies later, and tubules had less branch formation and proliferation when compared to healthy adults.[14] This is of clinical importance, since autologous ECFC use to treat ischemic conditions may not be advisable. Furthermore, ECFCs only circulate in very small numbers, therefore isolation and further expansion *in vitro* is necessary to reach therapeutic numbers.[104]

1.6.2 Equine regenerative angiogenesis

In equine medicine, cell therapy has been focused on the treatment of musculoskeletal conditions, primarily by the use of MSCs derived from adipose tissue or BM, while in human medicine, these therapies are also focused on immune-mediated, inflammatory and ischemic diseases.[105] Interestingly, there is strong evidence that the effects of MSCs are primarily paracrine by secreting immunomodulatory and trophic factors rather than direct differentiation into the desired cell type, and this paracrine effect may also stimulate blood vessel formation.[106, 107] This opens the idea of expanding cellular therapy in horses towards the use of ECFCs for the treatment of conditions affecting perfusion.

Ischemic diseases in horses, such as laminitis, wound healing, or colic, can lead to tissue degeneration and delayed healing, therefore it is key to identify therapies than can accelerate and improve vasculogenesis. In the case of laminitis, ischemia of the microvasculature of the laminal dermis has been implicated as an underlying cause in horses, especially for supporting limb laminitis.[108] By the use of Doppler ultrasound and near infrared spectroscopy, studies have demonstrated that weight bearing interferes with arterial perfusion of the foot, and when

prolonged for 48 hours or longer, there is increased expression of HIF-1α in the lamellae and increased lactate:pyruvate ratio in the lamellar dermis, which are suggestive of lamellar ischemia.[108, 109] Endocrinopathic laminitis (hyperinsulinemia associated laminitis) is considered the most common form of laminitis affecting equids. Although no clear relationship has been established between endocrinopathic laminitis and hypoperfusion in horses,[110] it is known that insulin signaling through insulin receptor will have an effect on lamellar perfusion, since the expression of this receptor seems to be restricted primarily to the microvasculature of the lamellae.[111] Although not investigated in horses, it is well known that humans with obesity or metabolic syndrome will have less number and dysfunction of ECFCs, predisposing them to cardiovascular disorders, therefore there is a potential benefit in the use of ECFCs as therapy.[112] Nevertheless, in horses there is an inverse relationship between fat mass and immunoreactive adiponectin concentrations.[113, 114] Inflammatory laminitis secondary to conditions such as colitis, proximal enteritis or pneumonia, among others, is thought to be caused by endotoxemia and subsequent systemic inflammation. In horses no studies have determined the relationship between inflammatory laminitis and laminar perfusion, however in the face of ischemia and reperfusion injury vascular dysfunction is well documented and it is largely a consequence of changes in ECs, affecting the cell-to-cell integrity, cytokine and adhesion molecule expression and vascular tone. Moreover, the release of inflammatory mediators initiate inflammatory and coagulation cascades that could occlude capillaries.[115]

Regardless of the cause, tissue damage, including local vascular damage to the foot lamellae, due to mechanical injury or inflammation are features of acute laminitis. Moreover, histologically, different studies have agreed in the observation of perivascular inflammation and

EC activation.[110, 116] All of this may suggest that ECFC dysfunction could be part of the pathophysiology of various types of laminitis and a possible therapeutic target.

Colic is the main cause of morbidity and mortality in horses, and it is estimated that in the United States there are about 4.2 colic events per 100 horses per year, with a fatality rate of 11%.[117] Strangulating obstruction will simultaneously occlude the intestinal lumen and vasculature, resulting in ischemic mucosal injury, which is the principal cause of colic-associated deaths in horses.[118] Furthermore, non-strangulating lesions can also lead to intestinal ischemia of various degrees. A non-strangulating obstruction, if not treated, could eventually create focal pressure in the mucosa leading to decreased perfusion within the intestinal wall.[119] There are no reports studying the use of ECFCs for the treatment of intestinal ischemic injury in humans or animal species. However, the effect of VEGF as a potential therapy for ischemic bowel disorders in humans has been evaluated, and it is thought to have a great therapeutical potential due to its ability to restore perfusion by increasing angiogenesis.[120]

Horses are prone to distal limb injuries partly because of their environment and partly due to their behavior in response to fright. Moreover, many of these skin wounds are not good candidates for primary surgical closure because of massive tissue loss, excessive skin tension, extreme contamination or delayed in medical attention, therefore there is a great likelihood that healing has to be by second intention.[121] Delayed or poor wound healing is a common problem in equine species, and especially if located in the distal limbs, can be a challenge to treat. Wound healing can be explained in 4 overlapping phases: hemostasis, inflammation, proliferation and remodeling. In the cases of horse's abnormal wound healing, the wound repair process will remain at the proliferative phase, where fibroblasts persist and synthesized

extracellular matrix instead of being eliminated. At the same time, the local ischemia and hypoxia often leads to an increase blood vessel sprouting and abundant vascular supply, thus inducing exponential growth of exuberant granulation tissue, also known as “proud flesh”.[122] This excess tissue formation and delayed wound healing results in poor functional and cosmetic outcomes, could potentially limit the horse’s performance career, as well as incur in excessive medical expenses for the owners. [121-123]

At the beginning of the wound healing process, appropriate oxygen supply is determinant to sustain the repair process, otherwise healing is impaired. In horses, it was observed that body wounds had higher concentrations of oxygen at the initial period of healing when compared to distal wounds, leading to a state of hypoxia during the inflammatory phase. This could explain the predisposition to delayed wound healing and formation of exuberant granulation tissue in distal limbs of horses.[122] Although it seems contradictory to have excessive formation of blood vessels in the phase of an impaired wound healing process, it has been observed that vessels within the granulation tissue are often dysfunctional in part due to occlusion secondary to EC hyperplasia and hypertrophy. Moreover, when compared to wounds in the thoracic region, distal limb wounds were 2.22 times more likely to have microvessel occlusion.[124]

Exuberant granulation tissue resembles the human keloid, and this has also been a challenge in human medicine, thus horses may serve as a human model for the treatment of abnormal wound healing in humans. This is due to the similarity of cellular processes underlying wound healing in both species, which occurs primarily through cellular proliferation and re-epithelization, rather than contraction.[123] The administration of ECFCs in horses suffering

from delayed wound healing and exuberant granulation tissue may help repair damaged blood vessels and improve perfusion in areas of tissue hypoxia.

A study was done where equine ECFCs encapsulated in polyethylene glycol-fibrinogen, a biomaterial that will protect the cells allowing for a more sustained release, were delivered locally at the periphery of wounds created in the distal limb of horses. The wounds were left to heal by second intention for 4 weeks. By the end of the study period, ECFC-treated wounds had smaller surface area and greater blood vessel density compared to non-treated wounds; moreover ECFCs were observed to incorporate into the host's vasculature by the use of cell quantum dot cell labelling.[125] However, no differences were observed in the degree of exuberant granulation tissue formation.[125]

1.7 Justification for study

Ischemic diseases in horses such as laminitis, delayed wound healing or colic are common in horses and could lead to early ending of their performance career, significant medical expenses, and even be life-threatening.[117] The use of cellular therapies to treat ischemic conditions is gaining research interest in human medicine, and have proven to be successful in restoring blood supply in mouse ischemic models.[126]

A cellular candidate to be used in regenerative angiogenesis are ECFCs, which have the unique characteristic of promoting angiogenesis both by paracrine and autocrine stimulation. These cells are able to form vessel-like structures *in vitro*, as well as repair and form *de novo* functional vessels *in vivo*. Although equine ECFCs have been successfully isolated from

peripheral blood of horses and locally delivered to distal wounds, the isolation techniques and analysis may have easily led to a mixed cell population. There are challenges to overcome when it comes to the phenotypic characterization and culture for expansion with equine ECFCs.[29, 125]

There is no consensus in the phenotypic characterization of ECFCs, or standardized isolation and culture protocols, and these cells are known to have a highly dynamic phenotype, changing based on tissue location, disease processes, and culture conditions.[7, 127] Current isolation protocols are not strictly selective on ECFCs, and other cell types such as monocytes and platelets can not only overgrow the ECFCs but also transfer proteins leading to phenotypic shift.[128, 129]

The use of molecular techniques for the selection of ECFCs based on EC phenotype has been successfully used when isolating human or mouse ECFCs. Therefore, selection of equine ECFCs based on expression of the EC surface marker CD31 or the ability to uptake Ac-LDL during the isolation and expansion process may result in a pure ECFC population. Different cell sorting techniques, based on magnetic selection or fluorescence signals can be used in equine cells to prevent cell contamination during expansion.

Traditionally, for ECFC expansion, cells are maintained with EGM that contains human growth factors, and although equine EGM is commonly supplemented with HS, other sources containing higher concentrations of equine-specific growth factors may be beneficial in stimulating the endothelial pathway towards preserving the ECFC phenotype. Equine platelet

lysate has been successfully used in the culture or equine MSCs; however, no studies have been done evaluating its effects in angiogenesis or ECFC culture as an alternative to the use of HS.

The use of angiogenesis function assays in conjunction with phenotypic characterization is necessary for the appropriate characterization of ECFCs. Equine ECFC function has been mostly assessed by tubule formation *in vitro*,[29, 31] however this does not take into account the interaction of supportive perivascular cells like its seen *in vivo*.[130] The use of *ex vivo* arterial models for angiogenesis has the benefit of better mimicking the *in vivo* processes involved in angiogenesis, and have been widely used to test the effect of diverse substances and cells in vessel formation and repair.[43, 130] The use of an *ex vivo* angiogenesis model using equine arteries will be useful in studying allogenic cell therapies and the use of equine-specific sources of growth factors like ePL to enhance angiogenesis or improve cell culture.

Additionally, there is a need to improve static cell culture of equine ECFCs to stimulate the signaling pathways towards the EC phenotype. *In vivo*, ECs are exposed to shear stress, and through cell membrane receptors and activation of downstream signaling the production of NO will be stimulated. Nitric oxide its known to have cytoprotective and cell proliferation properties, in part by increasing the secretion of VEGF.[81, 89] The addition of a NO donor to the cell culture media of equine ECFCs may overcome the limitation of static cell culture, preserving the phenotype and improving the angiogenic function and growth *in vitro*. The use of ECFCs to treat ischemic conditions in horses has great potential, however there is a need to improve isolation and culture conditions towards preserving their regenerative angiogenic capacity and phenotype.

Chapter 2 – Study aims and hypotheses

Specific aims

1. The isolation and phenotypic characterization of ECFCs faces many challenges not only because of the small number in circulation, but also since there is no consensus in their phenotype, and isolation of other unwanted cell types can overgrow or promote phenotypic shift of ECFCs. Therefore, the aim for the first part of the study was to identify EC surface markers expressed in isolated equine ECFCs and use either MACS to isolate a subpopulation of equine ECFCs with the $CD31^+$ phenotype, or FACS to isolate cells able to take Ac-LDL.
2. Evaluation of the angiogenic function of ECFCs is of utmost importance in the characterization of ECFCs since there are many inconsistencies in the use of phenotype alone. The use of *ex vivo* angiogenesis models is able to closely mimic *in vivo* models, with the advantage of being faster, reproducible and without the use of live animals. This aim focused on the evaluation of equine angiogenesis by the use of a modified mouse aortic ring assay, and its response to different blood-derived biologics as sources of growth factors, including HS, ePL and platelet-poor plasma (PPP).
3. Culture conditions are determinant in maintaining the desired phenotype and function of ECFCs, therefore it is important to enhance the EC pathway in cultured ECFCs during the expansion process. The aim of the third part of the study was to study the effects of ePL and the NO donor CysNO in *in vitro* function and phenotype of cultured equine ECFCs.

Hypotheses

1) Equine ECFCs will express the endothelial cell markers CD31, CD34 and CD105 by flow cytometry analysis. Thus, MACS based on expression of CD31 and FACS based on uptake of Ac-LDL will result in selection of an ECFC population that will maintain this phenotype once expanded.
2) Both a $CD31^+$ ECFC population and a cell population with high uptake of Ac-LDL will result in ECFCs with better in vitro angiogenic function when compared to ECFCs not previously sorted.
3) Equine facial artery rings will develop sprouting angiogenesis in culture when stimulated with endothelial growth media. The vascular network growth will be greater when adding ePL to the EGM as an equine-specific source of growth factors when compared to the use of HS or PPP.
4) The addition of the NO donor CysNO to the EGM for the expansion of equine ECFCs will enhance the EC phenotype. This will result in ECFCs with higher CD31 expression, greater growth kinetics and better in vitro angiogenesis when compared to the supplementation with EGM alone.

Chapter 3 – Sorting of equine endothelial colony-forming cells based on endothelial cell phenotype

3.1 Introduction

Endothelial colony-forming cells (ECFCs) are truly endothelial progenitor cells (EPCs) with capabilities of vascular repair by either paracrine and autocrine stimulation.[8] These cells have the ability to migrate to sites of ischemia, integrate with the host vasculature, form new blood vessels, and improve vascular function.[127, 131, 132] Endothelial colony-forming cells appear to be a promising therapy for regenerative angiogenesis and prevention of vascular damage in ischemic conditions in humans.[7] Similarly, cell therapy using ECFCs for regenerative angiogenesis have a potential to be used in animals such as the horse.

Horses are prone to ischemic conditions such as laminitis, delayed wound healing or ischemic colic, all of which are a big source of economic loss, have an impact in the horse's career, and can be life-threatening.[109, 117] For instance, many performance horses that suffer injuries in the distal limbs are at risk of delayed wound healing, where impaired angiogenesis and tissue hypoxia results in the growth of exuberant granulation tissue.[123] Likewise, considering that colic is the most common medical condition in horses, if complicated with decreased blood supply to the intestinal tract, is not only life-threatening, but can also significantly delay recovery and incur in great medical expenses.[117] Undoubtably, laminitis is one of the most serious, life-threatening conditions that can affect a horse's career and its quality of life, and current treatments are often unsuccessful. This is a condition where lamellar damage, changes in vascular dynamics, and the presence of hypoxia will lead to endothelial

dysfunction and decreased perfusion to the foot, ultimately causing tissue necrosis and detachment of the bone from the hoof wall.[133] Therefore there is a need for developing new cellular therapies that can promote vascular repair and *de novo* vessel formation to treat the different ischemic conditions affecting horses.

The therapeutic use of ECFCs has been limited, in part due to the inconsistent definition of these cells, the difficulty in their phenotypic characterization, as well as the high variability in isolation and culture protocols.[7, 134] These difficulties in phenotypic characterization of ECFCs are also present in animal species such as the horse, in part because of the lack of equine-specific endothelial cell (EC) surface antibodies. Isolation of equine ECFCs colonies with a typical cobblestone morphology has been done from peripheral blood of horses.[29] Moreover, these equine ECFCs were able to uptake acetylated low-density lipoprotein (Ac-LDL), form tubules in Matrigel® basement membrane and express the EC markers cluster of differentiation (CD) 34, CD105, vascular endothelial growth factor receptor-2 (VEGFR-2) and von Willebrand factor (vWF).[28, 29] It is important to note that only CD105 was successfully used for flow cytometry (FC); the other antibodies were only successful in indirect immunofluorescence, and once expanded in culture, a high expression of the hematopoietic cell marker CD14 was observed.[28]

Traditionally, human ECFCs have been characterized by the positive expression of the surface proteins CD31, VEGFR-2, and CD34, but most importantly, they lack expression of hematopoietic and monocyte markers CD45 and CD14.[7] However, there is an overlap in phenotype of ECFCs and some hematopoietic cells, since these cells may share some of the endothelial surface proteins.[23, 40] Additionally, overgrowth of other cell types during the

expansion phase following ECFC isolation is a common challenge described in human ECFCs, this in part due to the low number of cells in circulation, and hematopoietic cells attaching and invading the ECFC colonies once plated.[56]

These deficiencies in the isolation and expansion of ECFCs can impact the angiogenic function *in vivo*. The use of equine ECFCs was studied in distal limb wounds of horses, resulting in treated wounds with smaller surface area and greater blood vessel density compared to non-treated wounds; moreover, ECFCs were observed to incorporate into the host's vasculature.[125] However, some of these differences were not statistically significant, which could be attributed to deficiencies in cell phenotype and function. Hence, there is a need to improve isolation and culture conditions of equine ECFCs, in a way that the signaling pathways that drive their angiogenic potential are stimulated.

Flow cytometry analysis allows for the characterization of cells based on the expression of surface antigens, and it is one of the most common techniques used to phenotypically characterize ECFCs, both in humans and horses. In humans, the $CD31^+$ or the combination $CD31^+$ / VEGFR-2^+ phenotype is often used to characterize ECFCs due to the specificity as EC surface markers.[135, 136] This phenotype is desired for ECFCs with potential regenerative angiogenic functions, which has been supported in a study using mice, where treatment with human ECFCs lacking the expression of CD31 significantly decreased angiogenesis, but the presence of VEGFR-2 was related to the release of endothelial growth factors by these cells.[137] In order to select the desired phenotype for isolated ECFCs and produce a subpopulation with no contamination from other cell types, cell sorting techniques have gained interest to refine both isolation and expansion of human or mice ECFCs. Isolation of human

ECFCs has been done from peripheral blood by plating $CD31^+$ cells magnetically sorted until colonies developed for further expansion.[62] Additionally, fluorescence-activated cell sorting (FACS) based on the positive uptake of Ac-LDL, which is a desired function of ECs, has been used to isolate circulating ECFCs from rat whole blood.[58]

The use of cell surface proteins such as $CD31^+$ and VEGFR-2^+ to characterize and isolate equine ECFCs could result in a cell population with better function for future *in vivo* use, and this could be achieved by the use of cell sorting techniques. There are no reports of the use of live cell sorting for isolation and further refining of equine ECFCs based on the ability to uptake Ac-LDL or the expression of EC surface markers. Therefore, the aims of this study were to evaluate the expression of the surface EC markers CD31, CD34, CD105 and CD14 on equine ECFCs, and use magnetic-activated cell sorting (MACS) for selection of $CD31^+$ cells, both from peripheral blood mononuclear cells (PBMCs) and cultured ECFCs. An additional aim was the use of FACS to select an ECFC population with high uptake of Ac-LDL. It was hypothesized that equine ECFCs will have the $CD31^+$ / $CD34^+$ / $CD105^+$ / $CD14^-$ phenotype. Additionally, cell sorting based on positive expression of CD31, or high uptake of Ac-LDL will result in better *in vitro* angiogenic function when compared to ECFCs not sorted.

3.2 Methods

3.2.1 Endothelial colony-forming cells isolation and culture

Sources of ECFCs were either cryopreserved or newly isolated. Endothelial colony-forming cells were isolated from 4 healthy university-owned horses [American Quarter Horse

(n=4), ages 3-12 years] as previously described.[29] Briefly, animals were sedated using xylazine 0.4 mg/kg IV. An area of about 3x3 cm at the cephalic vein was clipped and aseptically prepared. Local anesthesia was provided with a subcutaneous block of 2% lidocaine. Using a 16 gauge needle, 50 mL of whole was collected from the cephalic vein into a syringe containing heparin at 10 IU/mL of blood. Blood was transported in ice and immediately processed in the lab.

Endothelial growth media (EGM-2 with Bullet Kit, Lonza, Visp, Switzerland) was prepared by mixing the growth factor-free endothelial basal media (EBM) with the Bullet Kit [the Bullet Kit contains human growth factors (fibroblast growth factor, VEGF, insulin-like growth factor-1 and epidermal growth factor)]. Finally, 10% horse serum (HS) was added to the EGM (EGM-HS), before filtering to remove solids, bacteria, or debris using a 0.45 μm cellulose acetate membrane filter. The fetal bovine serum provided from the manufacturer was not added.

Endothelial colony-forming cells isolation was performed by density gradient centrifugation (DGC) using a high-molecular-weight polysaccharide (Ficoll-Paque™ Plus Media, Cytiva, Marlborough, MA, USA). Under a laminar flow hood, samples were transferred to 50 mL conical tubes. Ten mL of blood was then diluted in 10 mL of a 1:1 mixture of EGM+HS and 1X phosphate buffer solution (PBS). The diluted blood was slowly transferred on top of 20 mL of Ficoll®. Tubes were centrifuged at 1,600 xg for 30 minutes (23°C, with no break at the end of centrifugation), and the buffy coat layer transferred to a new tube for centrifugation at 300 xg for 10 minutes. The cell pellet was resuspended in EGM+HS and transferred to a collagen-coated flask containing warmed EGM+HS.

Flasks were incubated at standard cell culture conditions (37°C, 5% CO_2 and 95% humidity). Media was changed at 24 hours and then twice weekly until the colonies were harvested. Once colonies were identified, they were counted and harvested. Following a gentle wash with PBS, TrypLE® cell dissociation agent (Gibco, Grand Island, NY, USA) was added and the flask incubated for 2.5 minutes. Detached cells were transferred to a tube and centrifuged at 200 xg for 5 minutes. The pellet was resuspended, and a cell count performed. Cells were transferred to a collagen-coated flask with warmed media at a density of 10,000-13,000 cells/cm^2. Once 80% confluent, cells were used for characterization between passages 1 and 3.

Endothelial colony-forming cells previously cryopreserved from 4 additional horses [American Quarter Horse (n=4), ages 6-14 years] were thawed by direct immersion in a 37°C water bath. Cell suspension was added to a collagen-coated flask prepared with warm EGM-HS. Eight hours after seeding, EGM-HS was removed, the flask was washed with 1X PBS and new EGM-HS added. Media was changed every 2-3 days and cells harvested at 80% confluency.

3.2.2 Matrigel® tubule formation

Matrigel® (BD Biosciences, Bedford, MA, USA) was thawed at 4°C overnight as per manufacturer's instructions. Following cell dissociation, separate cell suspensions, one per well of a 96-well plate were prepared by mixing 12,000 cells in 200 μL of EGM-HS. Matrigel® was maintained in ice, and using chilled pipette tips and well plate, 80 μL of Matrigel® was added to each well. The plate was incubated at 37°C, 5% CO_2 for 15-20 minutes to allow for

polymerization. Following this, the cell suspension was added on top and incubated. Samples were analyzed under the inverted microscope at 0, 5, and 24 hours for tubule formation.

3.2.3 Uptake of acetylated low-density lipoprotein

Endothelial colony-forming cells were seeded at a density of 8,000 cells/cm^2 into collagen-coated 24-well cell culture plates. Cells were incubated for 24 hours at standard cell culture conditions. Cell growth media was replaced with warmed EGM-HS containing Ac-LDL at a concentration of 50 μg/mL and incubated for 6 hours. Cells were dissociated with TrypLE™ and 2 wells combined for preparation for flow cytometry. Cells were washed twice in 1x PBS, resuspended in 1% bovine serum albumin (BSA) and filtered using a 40 mm cell strainer prior to flow cytometry analysis.

3.2.4 Expression of cell surface markers

Cryopreserved ECFCs from 4 different cell lines, passages 2-4 were seeded and expanded until they reached 80% confluent. Cryopreserved ECs previously isolated from equine carotid arteries and characterized by expression of CD34, CD105, vWF and VEGFR-2 served as a positive control. A total of $5x10^5$ cells per conditions were prepared for FC as previously described.[138] Cells were blocked in 10% HS for 45 minutes. CD105 monoclonal mouse anti-human (0.02 μg/μL), CD14 monoclonal mouse anti-horse (0.01 μg/μL), CD34 monoclonal anti-mouse (0.01 μg/μL), CD31 monoclonal mouse anti-human antibody (0.02 μg/μL), VEGFR-2 monoclonal mouse anti-human (0.01 μg/μL) or vWF polyclonal rabbit anti-human (0.015 μg/μL)

primary antibodies were added to the cells and incubated in a 100 μL volume at room temperature for 1 hour. Cells were washed with PBS prior to the addition of the secondary antibody. Alexa Fluor™ 488 goat anti-mouse secondary antibody was used for anti-CD105, anti-CD14, anti CD31, and anti-VEGFR-2 at a 1:400 dilution. Streptavicin DyLight™ 550 conjugated was used for anti-CD34 at a 1:400 dilution. Secondary antibody for anti-vWF was Alexa Fluor™ 488 goat anti-rabbit at a 1:400 dilution. After addition of the secondary antibodies, cells were incubated in a 100 μL volume at room temperature for 1 hour. Before FC, cells were filtered in a 40 μm cell strainer to create a single-cell suspension.

3.2.5 Cell viability and assessment of cell clumping – preliminary studies

Fluorescence-activated cell sorting requires a single cell suspension to increase efficiency, therefore (ethylenediaminetetraacetic acid) EDTA is often used in the buffer.[139] Due to the potential cytotoxic effect of EDTA, a preliminary study was done to assess the cell viability and percentage of single cells after using different concentrations of EDTA in the FC buffer. The FC buffer was composed of 25 μg/mL DNAse + 5 mM MgCl in 1% BSA. Cryopreserved ECFCs from one horse were prepared for FC as previously described. After dissociation using Accutase® (Innovative Cell Technologies, San Diego, CA, USA), cells were washed twice in PBS and treated with Ghost dye™ (Tonbo Biosciences, San Diego, CA, USA). For this, cells were resuspended in PBS containing 1 μL of Ghost dye™ and incubated at 4°C for 30 mins. Cells were washed with 1%BSA and treated with DNAse (100 μg/mL DNAse + 5 mM MgCl for 20 mins at room temperature). Cells were resuspended in 1%BSA and divided into 4 tubes

containing $5x10^5$ cells each. The 4 groups were defined based on the EDTA concentration in the FC buffer: 1) 0.5 mM EDTA, 2) 2mM EDTA, 3) 5mM EDTA, 4) Control (no EDTA). Each of the conditions was analyzed by duplicate. Percentage of single cells was determined by a two-step gating process as follows: 1) analysis of the cell population in forward versus side scatter allowed for removal of cell debris based on their small size and granularity, and 2) remaining cells were analyzed by forward scatter area versus forward scatter height, resulting in the gating of single cells of similar size.

Mean percentage of dead cells for the untreated (1.0 ± 1.2), 0.5 mM EDTA (1.0 ± 1.1), 2 mM EDTA (0.3 ± 0.1) and 5 mM EDTA (1.1 ± 0.1) were not statistically different (p=0.8198).

Mean percentage of single cells for the untreated (93.4 ± 4.1), 0.5 mM EDTA (92.6 ± 5.0), 2 mM EDTA (97.0 ± 0.9) and 5 mM EDTA (94.1 ± 1.5) were not statistically different (p=0.6119).

Even though differences were not significant, and the sample size was small, it was decided to use EDTA at a concentration of 2 mM for the remaining of the study since this resulted in the lowest mean percentage dead cells and the highest percentage of single cells.

3.2.6. Fluorescence-activated cell sorting cell preparation

Cryopreserved ECFCs previously deemed positive for Ac-LDL uptake using FC and confirmed to form tubules in a Matrigel® matrix were used. About $1.5x10^6$ cells were included in each of the cell sortings. Different protocols (described below) were tested to further reduce

cell clumping and optimize the yield. Cells from the different protocols were analyzed by FC to estimate the percentage of single cells.

Cells were analyzed in a Beckman™ flow cytometer. A minimum of 10,000 events were analyzed. For analysis, cells were gated to remove debris, doublets and dead cells when live/dead analysis was performed.

Protocol 1: 1% BSA

The standard protocol for cell preparation for FC analysis was used. Briefly, once cells were 70% confluent, they were supplemented with EGM-HS containing Ac-LDL at a concentration of 10 μg/mL. Cells were incubated and protected from light for 4-6 hours. Following incubation with Ac-LDL, cells were dissociated using TrypLE™ as previously described. The cell pellet was resuspended in PBS and washed twice. Following this, cells were resuspended in 1 mL of 1% BSA in N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) 25 mM and 5 mM MgCl buffer and filtered using a 40 μm cell strainer.

Protocol 2: 1% BSA / EDTA at 0.5 mM, 2 mM and 5mM

Cells were exposed to Ac-LDL as previously described. After cells were dissociated with TrypLE™ and washed in PBS twice, they were resuspended in 1% BSA containing either 1 mM, 2 mM or 5 mM of EDTA. Cells were filtered as previously described before cell sorting.

Protocol 3: 1% BSA / DNAse

Cells were incubated for 6 hours with EGM-HS containing Ac-LDL before being dissociated using Accumax® solution (Sigma-Alrich, St. Louis, MO, USA). Cells were washed in 1% BSA and incubated at room temperature for 20 mins in a DNAse solution (20 mL DNA + 5 mM MgCl + 1% BSA). Cells were washed in 1% BSA and resuspended in 1% BSA for sorting.

Protocol 4: 1% BSA / Accumax®

Cells were incubated for 6 hours with EGM-HS containing Ac-LDL. Following this, they were dissociated from the flask using Accumax®. Cells were washed twice with PBS and incubated for 20 mins at room temperature in a 1:1 suspension of 1% BSA and Accumax®. Cells were washed in 1% BSA and resuspended in 1% BSA for sorting.

Protocol 5: 1% BSA / EDTA / DNAse

Cells were incubated for 6 hours with EGM-HS containing Ac-LDL. Following this, they were dissociated from the flask using Accumax®. Cells were washed in 1% BSA and then incubated at room temperature for 20 mins in DNAse solution (20 mL DNA + 5 mM MgCl + 1% BSA). Cells were washed in 1% BSA and resuspended in the sorting media (1% BSA + 25 mM HEPES + 5 mM MgCl, + 2 mM EDTA + DNAse 25 mg/mL).

Protocol 6: 1% BSA / EDTA/ DNAse / Accumax®

Cells were incubated for 6 hours with EGM-HS containing Ac-LDL. Following this, they were dissociated from the flask using Accumax®. Cells were washed twice with PBS and incubated for 20 mins at room temperature in a 1:1 suspension of 1% BSA and Accumax®. Cells were washed in 1% BSA and resuspended in the sorting media (1% BSA + 25 mM HEPES + 5 mM MgCl + 2 mM EDTA + DNAse 25 mg/mL + Accumax® 0.5 mL). Cells were filtered prior to FC analysis.

3.2.7 CD31 antibody validation and titration

Prior to the study, CD31 mouse monoclonal antibody (Anti-CD31 antibody JC/70A Abcam) was tested in cryopreserved equine carotid ECs to confirm reactivity. Endothelial cells were thawed and expanded in a collagen-coated flask as previously described. Once 80% confluent, cells were dissociated with TrypLE™, centrifuged and resuspended in 10% HS in PBS for blocking at room temperature for 1 hour. Cells were washed with PBS and $3x10^5$ cells transferred to each of the tubes needed for FC. Prior to adding the primary antibody, cells were treated with Ghost Dye™ (Tonbo biosciences) for live/dead analysis. The primary antibody (CD31) was prepared at a concentration of 2 μg/100 μL, and cells were resuspended in the 100 μL solution for 45 minutes at room temperature. Cells were washed 3 times in PBS and resuspended in 100 mL of diluted secondary antibody Alexa Fluor™ 188 goat anti-mouse at a 1:400 dilution and incubated at room temperature for 45 minutes. Cells were washed 3 times in PBS, resuspended in 1mL BSA and filtered using a 40 μm cell strainer.

To find the appropriate CD31 antibody concentration for the MACS, titration was performed. Equine ECs were prepared as described above for FC. The primary antibody CD31 was diluted at concentrations of 4 μg/100 μL, 2 μg/100 μL, 1 μg/100 μL, 0.5 μg/100 μL, 0.25 μg/100 μL and 0.125 μg/100 μL.

3.2.8 Magnetic activated cell sorting

Endothelial colony-forming cells were isolated from whole blood of 4 healthy university-owned horses using density gradient centrifugation after cephalic venipuncture. The obtained PBMCs were either directly sorted or cultured for isolation of ECFCs colonies.

Positive selection MACS was done as per manufacturer's instructions (MACS Miltenyi Biotec). The cell pellet of either PBMCs from buffy coat or culture-expanded ECFCs were resuspended in a sorting buffer (0.5% BSA in PBS + 2 mM EDTA). After 2 washes in the sorting buffer, cells were resuspended in 10% normal goat serum in PBS for blocking. CD31 primary antibody was diluted in 100 mL of PBS at a final concentration of 2 μg/100 μL. After 1 hour incubation period, the cells were washed in the sorting buffer, filtered using a 40 μm cell strainer, and incubated for 15 minutes at 4°C in a 100 μL solution of anti-mouse IgG1 MicroBeads (Miltenyi Biotec). For the positive selection magnetic separation, the cell suspension was deposited into the magnetic column while attached to the magnetic field (QuadroMACS™ separator, Miltenyi Biotec). Sorting buffer was used to rinse the column, and the eluted $CD31^-$ fraction was collected. Following, the column was removed from the magnetic

field and rinsed with buffer to obtain the $CD31^+$ fraction. Finally, cells were counted and subcultured in collagen-coated well plates.

3.2.9 ECFC-sorted characterization

MACS was performed in PBMC from buffy coat following DGC from 4 different horses. Following cell sorting, cells for the $CD31^+$ and $CD31^-$ groups were seeded in collagen-coated well-plates at a density of 5,000 cells/cm^2. Cells were observed daily for attachment, proliferation, and formation of colonies. After 24 hours, EGM-HS was replaced to remove unattached cells, and media was changed every three days until cells were 80% confluent.

Following isolation, PBMC from buffy coat were also used for standard isolation as previously described. Cells were seeded in a collagen-coated T75 flask, EGM-HS was replaced after 24 hours and twice weekly thereafter until colonies appeared. Colonies were harvested and cells were either used for MACS or subcultured for expansion and sorting at later passages.

Once expanded, sorted cells were prepared for FC to measure expression of CD31, as well as live/dead analysis. Cells were also analyzed for tubule formation in Matrigel®.

3.2.10 Statistical analysis

Data was analyzed using GraphPad Prism 9.4.0. For the live/dead analysis, descriptive statistics was recorded. Percentages of dead cells, as well as number of singlets were compared by simple analysis of variance (ANOVA) with Tukey post hoc test. Results are reported as mean

± SD. Efficiency of each of the cell sorting and number of sorted cells within each subpopulation was recorded. Uptake of Ac-LDL and expression of CD31 in sorted cells was compared by unpaired t-test with Welch's correction.

3.3 Results

3.3.1 ECFC isolation

Following DGC from peripheral blood in 4 horses, one of the horses (horse 3) did not form colonies in culture. The culture of PBMCs following DGC resulted in colony formation for the remaining horses 2, 3 and 4. The first evidence of colony formation was observed by day 6. Colonies were harvested between days 9 and 13, for an average of 23 colonies per T75 flask. Morphology of the cells in the colonies was predominantly mixed, containing both cobblestone and spindle-shaped cells (Figure 3).

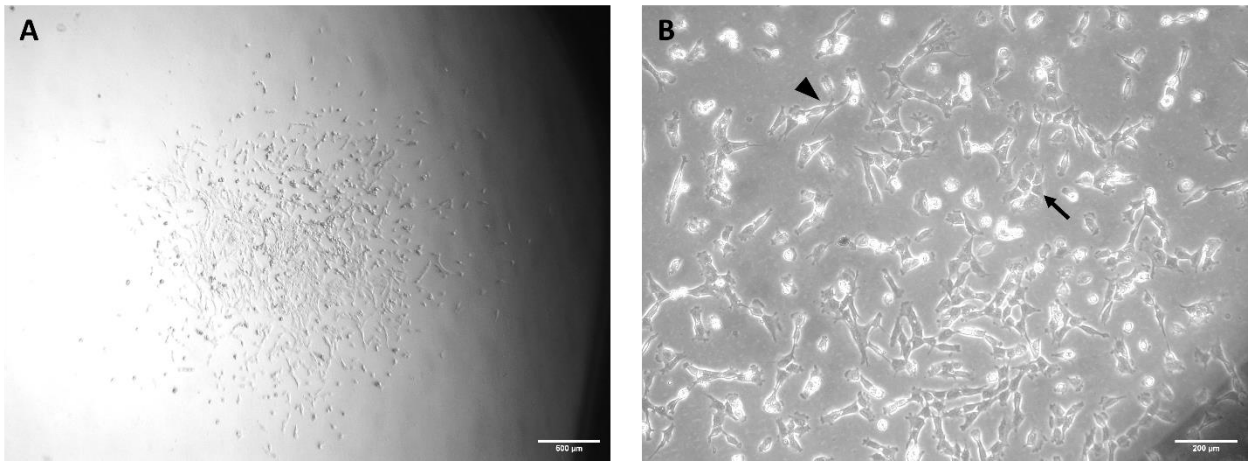


Figure 3. Equine ECFCs morphology. Photomicrograph of A) an entire ECFC colony at day 6 of culture after isolation from PBMCs following DGC from peripheral blood (bar = 500 μm), and B) cell morphology of the same ECFC colony at a higher magnification. Note the mixed cell morphology of both cobblestone (arrow) and spindle-shaped (arrowhead) (bar = 200 μm).

3.3.2 Cell surface markers

Equine carotid ECs analyzed by FC for CD31 expression demonstrated to be reactive to the monoclonal mouse anti-human CD31 antibody used, based on a mean expression of 64.41%. In contrast, the mean CD31 expression on cryopreserved equine ECFCs was 1.63% (Figure 4). The results from FC of the cell surface markers analyzed from 4 different ECFC lines previously isolated and cryopreserved are summarized in table 1.

Cell marker	% Expression ECFC (horse number)	% Expression carotid EC
CD105	96.73 (1)	
CD34*	0.62 (2)	1.27
CD14	99.55 (2)	77.63
VEGFR-2*	1.34 (2)	1.94
vWF*	1.2 (2)	3.46
CD31	0.81 (1)	56.23
	1.86 (2)	66.59
	1.76 (3)	
	2.1 (4)	

Table 1. Cell surface marker expression by flow cytometry in 4 different lines of cryopreserved equine ECFCs and EC. * denotes that the antibody was not optimized for flow cytometry by the manufacturer. CD105 was not tested in equine carotid ECs.

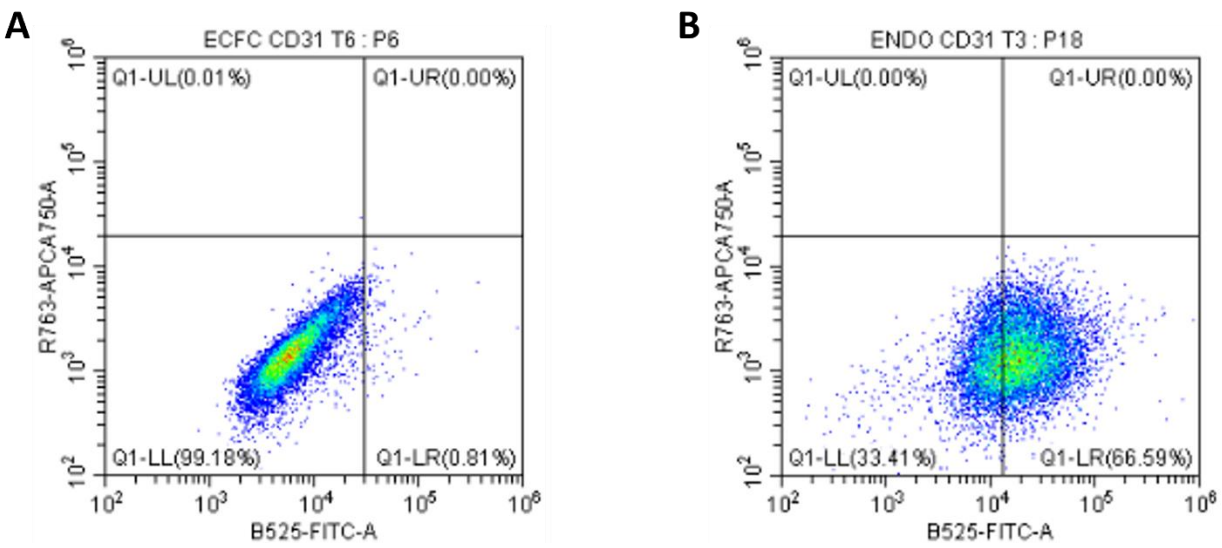


Figure 4. Expression of the endothelial cell marker CD31 in A) equine ECFCs and B) Equine carotid ECs by flow cytometry. Cells positive for CD31 uptake are represented in the right lower quadrant as single live cells after gating. The x axis corresponds to the fluorescence intensity of CD31 and the y axis the fluorescence intensity of Ghost Dye™.

3.3.3 Fluorescent-activated cell sorting

After testing the different protocols for creating a single cell suspension of ECFCs, protocols 1-4 resulted in excessive clumping and the FACS had no cell yield, clogging the nozzle of the sorter. Therefore, protocols 5 and 6 were used for FACS of ECFCs based on the uptake of Ac-LDL. A total of 4 FACS were done from cryopreserved cells of 3 different horses (Figure 5). The results of the FC analysis for determination of percentage of single cells following protocols 1, 5 and 6 are presented in the appendix A.

Each cell sorting was done with $1.5x10^6$ cells. Two sortings using protocol 5 had efficiencies of 37% and 40%. The other 2 cell sortings using protocol 6 had efficiencies of 94% and 70%. Results from FACS are summarized in table 2.

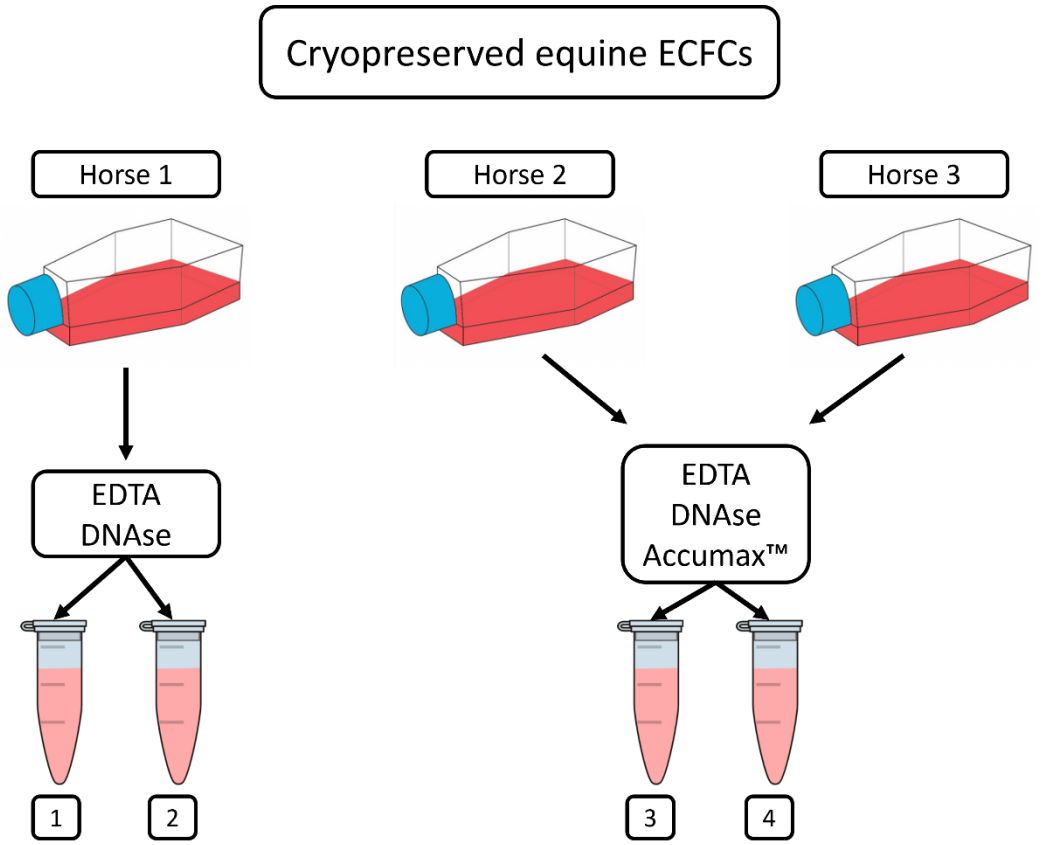


Figure 5. Representation of the study design for FACS using protocols 5 (2 mM EDTA + DNAse in 1% BSA) and 6 (2 mM EDTA + DNAse + Accumax™ in 1% BSA). Two sortings were performed per protocol, and 3 different cell lines were used.

		LDL-positive		LDL-negative	
Protocol	**Efficiency**	**Sorting yield (number of cells)**	**Post culture LDL uptake**	**Sorting yield (number of cells)**	**Post culture LDL uptake**
5	37%	17,200	98.69%	31,700	99.26%
5	40%	249,751	98.69%	131,190	99.26%
6	94%	227,000	92.57%	17,500	88.99%
6	70%	131,000	97.05%	219,000	96.79%

Table 2. Results from FACS of equine ECFCs. Protocols 5 and 6 were successful in sorting ECFCs. Percentage efficiency corresponds to the proportion of sorted cells out of the number of target cells to sort. The table shows, for each sorted population, its correspondent percentage uptake of Ac-LDL after expansion in culture. Note the positive uptake for ECFCs that were originally sorted based as being negative for Ac-LDL uptake.

The positive and negative cells were expanded in culture and analyzed for Ac-LDL uptake and Matrigel® tubule formation. Although the tubules were not high quality in any of the groups, LDL-positive cells maintained their *in vitro* tubule formation, while tubule formation was absent in LDL-negative ECFCs (Figure 6).

The percentage of Ac-LDL uptake after sorting was not significantly different between LDL-negative (96.1 ± 4.9%) and LDL-positive (mean 96.8 ± 2.9%) populations (P=0.8194) (Figure 7).

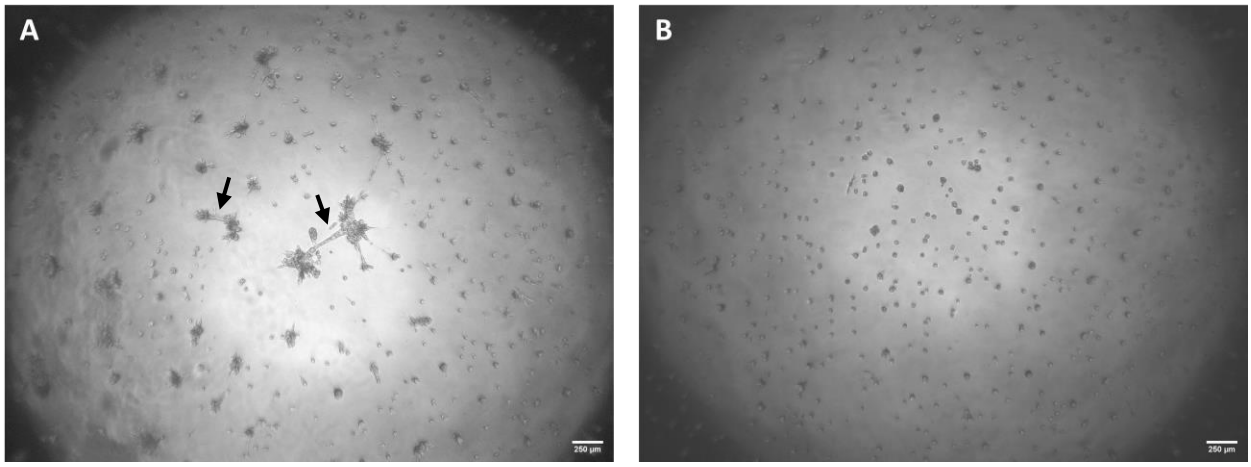


Figure 6. Matrigel® tubule formation of sorted equine ECFCs based on uptake of Ac-LDL at 24 hours. A) LDL-positive ECFCs showing evidence of connecting microtubules (arrows) and B) LDL-negative ECFCs with no evidence of microtubules. Photomicrographs were taken at 24 hours of culture in Matrigel®. Bar = 250 μm.

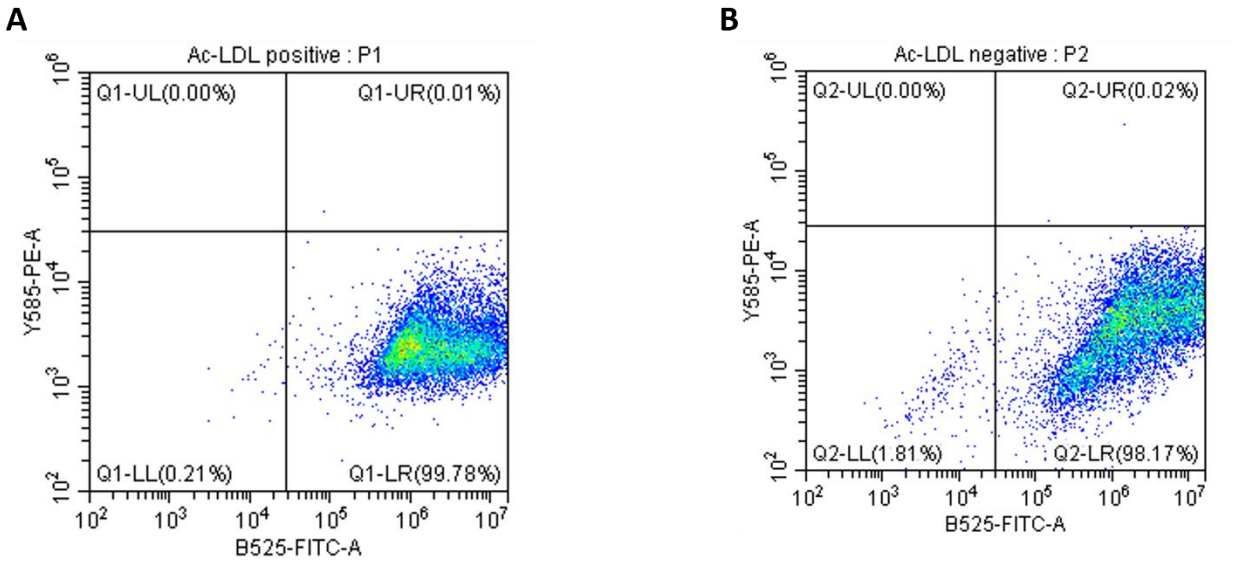


Figure 7. Ac-LDL uptake after expansion of sorted ECFCs. Flow cytometry analysis showing percentages of Ac-LDL uptake following expansion of sorted A) positive Ac-LDL and B) negative Ac-LDL equine ECFCs. The x axis corresponds to the fluorescence intensity of Ac-LDL positive cells.

3.3.4 Magnetic-activated cell sorting

MACS was performed in PBMCs from buffy coat following DGC from 4 different horses. Additionally, buffy coat was also cultured for ECFC isolation, and out of these 4 horses, 3 formed colonies, and cultured ECFCs were magnetically sorted at different passages (1 to 4) once expanded. Results from the MACS, both directly from buffy coat and from cultured ECFCs are summarized in table 3. Additionally, only for horse 1, subculture of $CD31^-$ cells sorted from PBMCs resulted in attachment to the flask and formation of colonies. CD31-sorted cells from buffy coat resulted in no cell attachment for the other horses. One of the horses (horse 3) did not form colonies, therefore it was withdrawn from further studies. A total of 5 MACS from ECFCs expanded in culture were done.

Following cell sorting and expansion, Matrigel® tubule formation, morphology in culture, and CD31 expression were assessed. For horse 1, both $CD31^+$ and $CD31^-$ sorted cells at passages 1 and 4 had a mixed morphology of cobblestone and spindle-shapes (Figures 8A and 8B). For horses 3 and 4, both $CD31^+$ sorted cells at passages 1, 2 and 4 had a mixed but predominantly cobblestone morphology (Figure 8C). Overall, $CD31^-$ cells presented a more mixed morphology with both spindle-shape and cobblestone cells (Figure 8D). All sorted cells, from both groups formed small microtubules in Matrigel®.

CD31 expression for the $CD31^+$ and $CD31^-$ sorted cells was measured for horses 1 and 4. Cells from horse 2 had bacterial contamination, therefore they were removed from the study before any FC analysis could be done. The mean CD31 expression in $CD31^+$ and $CD31^-$ sorted

cells was 7.56 ± 3.83 and 6.04 ± 2.84 respectively (Figure 9). The difference in CD31 expression between $CD31^+$ and $CD31^-$ cells was not significant (P=0.7283).

Source	PBMCs	Cultured ECFCs
Mean total sorted cells	$33.8x10^6$	$7.79x10^6$
Mean $CD31^+$ yield	$3.63\ x10^5$	$22.69x10^6$
Mean $CD31^-$ yield	$1.39\ x10^5$	$4.57x10^6$

Table 3. Sorted cells and yields based on CD31 expression. Magnetic-activated cell sorting was performed from the PBMCs fraction or cultured ECFCs. For each of the cell sources, the total mean sorted cells and its respective $CD31^+$ and $CD31^-$ subpopulations are provided.

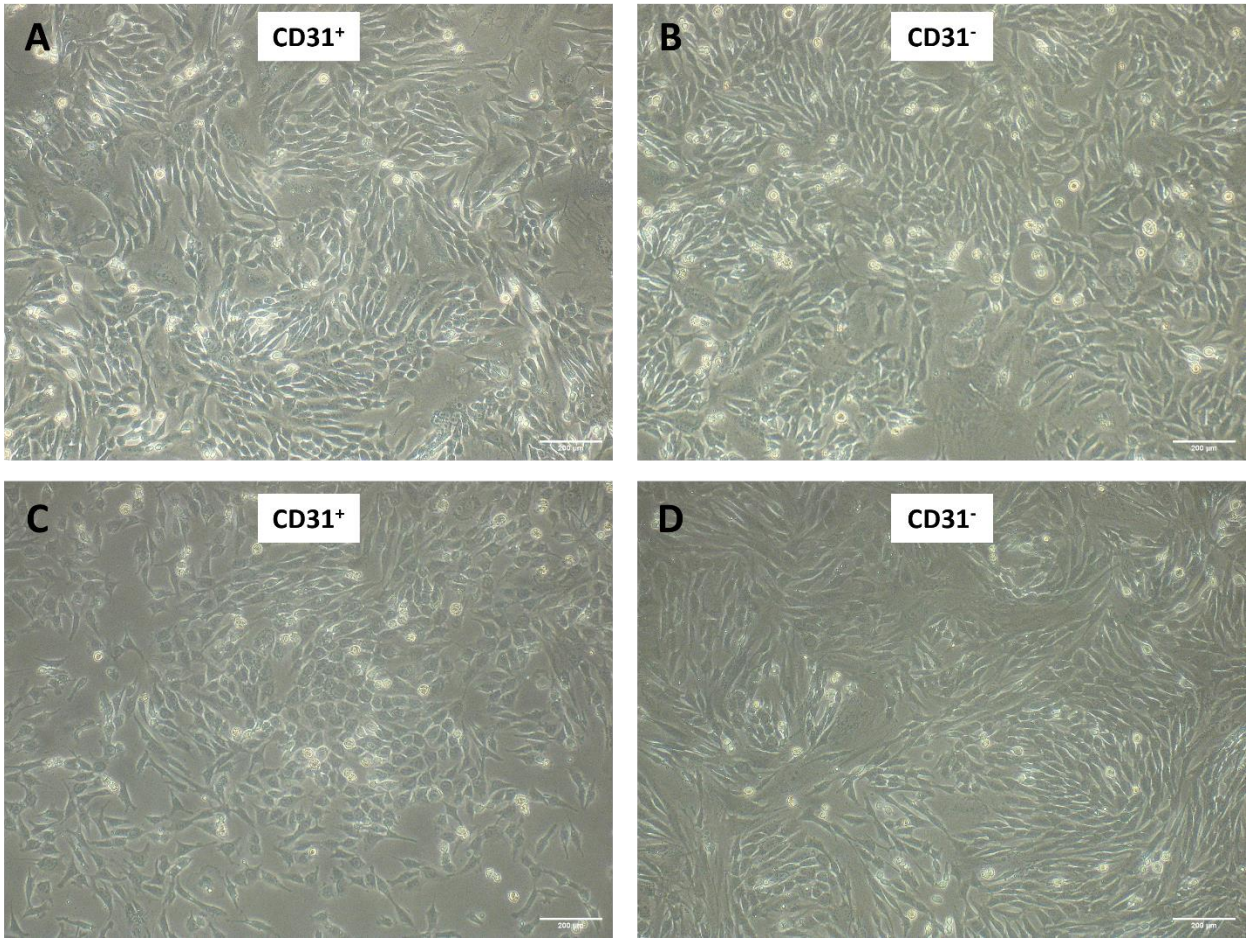


Figure 8. Cell morphology of cultured equine ECFCs magnetically sorted based on CD31 expression. A) and B) correspond to ECFCs from horse 1, showing a mixed morphology for both the $CD31^+$ and $CD31^-$ groups. C) corresponds to an expanded subpopulation of $CD31^+$ sorted ECFCs from horse 3 at passage 2, where a more uniform cobblestone morphology is observed, compared with D) a subpopulation of $CD31^-$ sorted ECFCs from horse 4, which displays a mixed morphology consisting of both cobblestone and spindle-shaped cells. Bar = 200 μm.

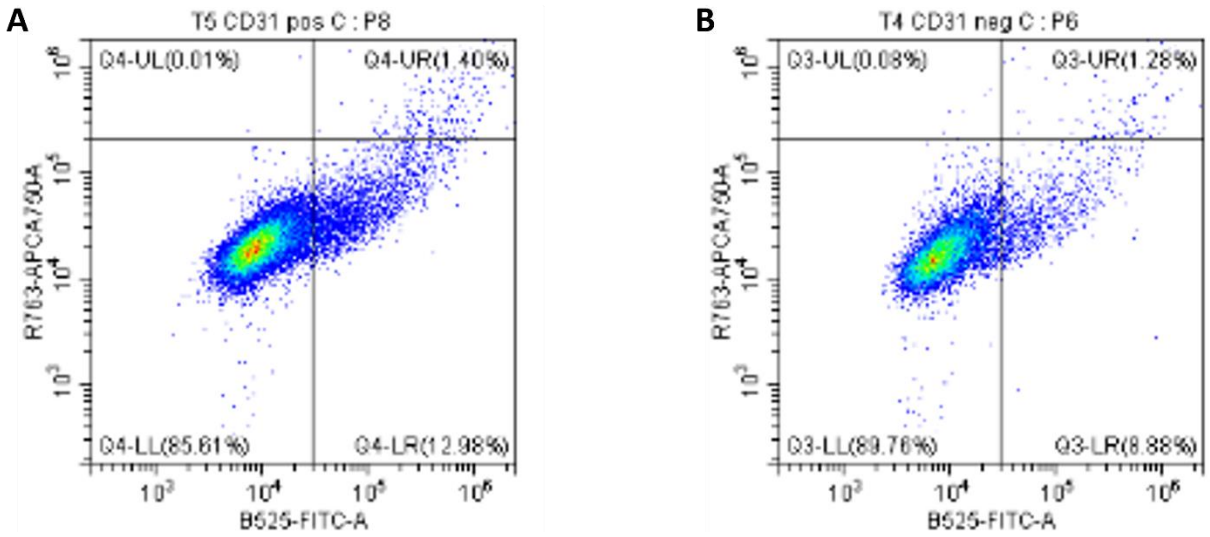


Figure 9. Percentage CD31 expression of sorted equine ECFCs after expansion in culture. Flow cytometry results of ECFCs magnetically sorted initially as A) $CD31^+$ and B) $CD31^-$. Percentage of positive cells in the right lower quadrant represent single live cells after gating. The x axis corresponds to the fluorescence intensity of CD31 and the y axis the fluorescence intensity of Ghost Dye™.

3.4 Discussion

Equine ECFCs were successfully isolated from peripheral blood as previously described,[29] and although the isolated cells were proliferative, formed tubules *in vitro* and were able to uptake Ac-LDL, most of the colonies were observed to be of mixed morphology instead of predominantly cobblestone. The mixed morphology observed in the colonies raised concerns about obtaining a mixed cell population, rather than a pure subset of ECFCs. This is a well-known issue when isolating a rare cell population, which is the case of circulating ECFCs. The isolation technique used in this study is based on the attachment of ECFCs to the collagen-coated plate, and further discard of unattached cells. Moreover, once expanded, myeloid angiogenic cells may also proliferate and can overgrow the cells of interest. Even though these cells isolated from equine peripheral blood showed angiogenic capabilities, characteristic of the intrinsic vasculogenic ability of ECFCs, paracrine stimulation from myeloid angiogenic cells could enhance *in vitro* tubule formation, and similarly, cells from the hematopoietic line are able to uptake Ac-LDL.[23] Moreover, ECFCs could shift phenotype in response of paracrine stimulation from other cell types.[140]

The appearance of colonies on day 6 was consistent with a previous report using DGC isolation from cephalic veins in horses.[29] This early colonies, appearing between 4-7 days in culture, and displaying predominantly a spindle shape morphology, could correspond to early EPCs.[13]

Previously used endothelial antibodies for characterization of equine ECFCs and ECs were used for immunofluorescence on fixed cells that could no longer be sorted and

subcultured.[29, 138] In this study, antibodies against CD34, VEGFR-2 and vWF were evaluated by FC with the purpose to be used as markers for FACS of live cells; however, these commercial antibodies were not validated to be used by FC. Therefore, it was not surprising that both equine ECFCs and ECs were negative, since these antibodies recognize linear epitopes that are only accessible when denature like its done in immunofluorescence, while FC is based on the analysis of whole cells and their proteins in their native form.[141] Another factor limiting with the use of specifically vWF was the intracellular location of this protein within the Weibel-Palade bodies, and most of the protocols for FC will require permeabilization for intracellular staining, which was not done in our case since the purpose was to evaluate live cells for future cell sorting.[142]

In this study it was demonstrated that live equine ECFCs can be sorted based on the expression of the surface protein CD31 or the uptake of Ac-LDL. Most importantly, these cell sortings yielded cell subpopulations that were able to be expanded *in vitro*.

Magnetic activated cell sorting provides a sorting technique that is more accessible and faster, therefore reducing the time that cells are outside their culture environment. The fact that after expansion, $CD31^+$ sorted cells had a very low expression of CD31 could be explained by the low number of cells obtained. Such low cell yield will require more passages to expand before they can be analyzed by FC, increasing the risk for overgrowth of other cell types. Furthermore, MACS often results in a cell purity after separation that is lower when compared to FACS because of non-specific binding of the magnetic beads. Additionally, cell clumps will not be “rejected”, and cells of different types that are not in a single suspension can be eluted together.[143]

Even though human placenta ECFCs have been successfully isolated based on CD31 expression, colonies were observed to have mixed morphology, reinforcing the fact that further purification techniques, like additional cell sortings, might be required.[56, 144] In this study it was not possible to successfully expand the $CD31^+$ cell fraction from the buffy coat, which has been reported to be possible when isolating $CD31^+$ cells from human peripheral blood following DGC.[62] This could be due to the low cell number recovered in addition to possible damage to the cells secondary to the mechanical forces applied during magnetic separation.[145] Interestingly, in the present study, ECFCs magnetically sorted after expansion in culture did attach to the culture flask and propagated successfully, however the yield resulted in a greater cell count when compared to magnetic sorting directly from the buffy coat.

Following expansion, sorted ECFCs from both the $CD31^+$ and $CD31^-$ fractions shared similar CD31 expression and formed microtubules *in vitro*, suggesting that a similar cell population was arising in both groups. Interestingly, in some cell lines where sorting was done at lower passages (for example in horse 3 at passages 1 and 2), the $CD31^+$ fraction resulted in cells with more cobblestone morphology, which is characteristic of the endothelial phenotype. This may suggest that even though the CD31 phenotype was not conserved at later passages, it is possible that at lower passages after MACS the $CD31^+$ phenotype was predominant. Unfortunately, on horse 3, where MACS was done at lower passages and the $CD31^+$ presented a cobblestone morphology, FC analysis was not performed due to sample contamination. However, very low CD31 expression was a common finding in equine ECFCs from this study, suggesting that either phenotypic shift during culture due to paracrine stimulation of different cell types, or isolation and culture protocols are favoring other cell types that will overgrow the

ECFCs. For instance, when human ECFCs are co-cultured with smooth muscle cells, the expression of mesenchymal cell marker increases concomitantly with a decrease in CD31 expression.[146]

An unexpected result was the fact that a high expression of the myeloid cell marker CD14 was observed in equine carotid ECs. However, expression of CD14 has been documented in human umbilical cord ECs, as well as in EC from the iris, cornea and liver sinusoids, which could be evidence that the CD14 expression is not restricted to cells of the myeloid lineage.[147]

Endothelial cells are prone to clump together and clog the sorter nozzle, making FACS difficult. In this study, by modifying current general cell sorting protocols, equine ECFCs were successfully sorted using FACS when dissociated with Accumax™ and a combination of EDTA and Accumax™ are used as part of the sorting buffer. A combination of 2 mM of EDTA and Accumax™ in the sorting buffer was beneficial in increasing the number of single cells when compared to BSA alone or even BSA and EDTA. ECFCs treated with this combination displayed greater sorting efficiency, meaning that this treatment was able to create a single cell suspension that remained non-clumped during the sorting process. Efficiency is a measure of sorting performance, and it is defined as the number of target cells sorted divided by the number of target cells detected.[148] EDTA has been used to reduce cell clumping when sorting cells, however at higher concentrations it can cause cytotoxicity by increasing osmolality and promote water excretion from cells.[149] Single cell suspensions are ideal for cell sorting procedures, not only for an accurate cell count before and after the procedure, but for improvement of efficiency and purity of the selected cell population. Accumax™, a natural

enzyme mixture with proteolytic and collagenase activity is advertised for cell dissociation and creating a single cell suspension, making it a good candidate for cell counting and efficient cell sorting. The ability of Accumax™ to dissolve cell clumps and produce a single cell suspension has been tested in human ECs prior to FACS.[150] This study demonstrated that Accumax™ can be safely used in equine ECFCs to create a single cell suspension suitable for FACS.

Even though a positive and negative cell population based on Ac-LDL uptake was successfully obtained, after being expanded in culture, both subpopulations were able to uptake Ac-LDL. Although unexpected, this result is consistent with the fact that other cell types, such as monocytes, mature ECs and other bone marrow-derived cells are able to uptake Ac-LDL.[32] Similarly, cultured MSCs have also been reported to uptake LDL when undergoing differentiation.[151, 152] Therefore, Ac-LDL uptake by previously negative sorted cells might be related to other cell types overgrowing, as well as originally LDL-positive ECFCs filtered in the negative fraction. Even though the Ac-LDL uptake was inconsistent in separating cell groups after expansion, sorting based on Ac-LDL uptake might have a positive effect in functionality based on better tubule formation *in vitro*. This could lead to further studies using Ac-LDL in combination to other EC markers for more efficient cell sorting.

Although it was demonstrated that both MACS and FACS are possible in equine ECFCs, some limitations need to be overcome in order to further use this technique in the isolation of ECFCs. Current culture techniques might be favoring the attachment of other cell types such as platelets, and cultured hematopoietic cells can acquire an EC phenotype by passive transfer of platelet microparticles containing CD31, thus misclassifying cells of the myeloid lineage as ECs.[25] Ac-LDL uptake, although known to be a function of ECs, is not specific to progenitor

ECs, and it can be used to characterize mature ECs or other cell types such as macrophages.[18] Therefore, the sole expression of CD31 and uptake of Ac-LDL are not sufficient to define an endothelial progenitor cell *in vitro*, and the combination with other cell markers as well as assessing functionality *ex vivo* or *in vivo* are needed. However, the lack of equine-specific endothelial antibodies to use by FC is a limitation when using sorting techniques, since a live cell population is needed for expansion.

This study used cells sorting techniques not previously described in equine ECFCs. Although results were conflicting, cell sorting techniques are viable, however a proper protocol for cell separation to guarantee a single cell suspension is critical. Furthermore, refining of current isolation and culture techniques towards reducing contamination from other cell types, as well as the addition of alternative equine-specific vascular growth factors may help improving the use of cell sorting technique in equine ECFCs.

Chapter 4 - Horse serum or equine platelet lysate increases total vascular endothelial growth factor A concentrations and correlates with vascular growth in an equine facial arterial ring assay

4.1 Introduction

Angiogenesis is defined as the growth of new blood vessels from pre-existing ones, in a process that involves endothelial and supporting cells, all guided by pro-angiogenic growth factors that will stimulate the appropriate signaling pathways.[130] Horses are known to suffer from ischemic conditions such as laminitis, where dysfunction of the microvasculature of the laminal dermis has been implicated as an underlying cause.[108] Similarly, other ischemic conditions such as delayed wound healing or some forms of colic can have severe implications in their career or be life-threatening. Because of this, research has been done on identifying cell-based therapies that can accelerate and improve angiogenesis in horses, however, most of the studies have been done *in vitro*.[29, 153] Endothelial colony-forming cells (ECFCs), a type of endothelial progenitor cell, have the important function of stimulating blood vessel formation and repair, making them good candidates to study cell-based therapy in horses.[39] However, the lack of consensus in their phenotypic definition makes *in vitro* studies difficult to interpret.[40] Since angiogenesis involves many cellular and molecular processes, *in vivo* studies are warranted, but this comes with the limitations of being more expensive, invasive, often times difficult to analyze, and may raise ethical concerns.[39]

Ex vivo models of angiogenesis using vascular explant cultures are useful in evaluating angiogenesis because they overcome the limitations of *in vitro* techniques, while reducing the complexity of *in vivo* models, thus bridging the gap between these techniques.[41, 42] E*x vivo* models are able to mimic most of the steps of angiogenesis seen *in vivo*, such as initial sprouting, matrix remodeling and lumen formation. Paracrine signaling between endothelial cells, fibroblasts, macrophages and pericytes are critical in formation of vascular tubes, which can be mimicked in *ex vivo* assays.[42, 43] This signaling can be stimulated by equine-specific angiogenic growth factors, such as vascular endothelial growth factor A (VEGF-A). Fetal bovine serum (FBS) is widely used to supplement endothelial growth media (EGM), however its xenogeneic origin increases the risks for immune reactions, and there are ethical concerns that arises from its harvesting.[66, 154-156] When culturing equine ECFCs, horse serum (HS) have been successfully used as a supplement of EGM for cell expansion.[28, 29] However, studies in humans are contradictory in the efficacy of human serum/plasma for expansion of endothelial cells.[66]

Human platelet lysate is a rich source of growth factors, including VEGF-A, and has been used for propagation of different cell types, including endothelial cells. In human cultured ECFCs, platelet lysate enhanced the formation of vascular structures both *in vitro* and *in vivo*.[67] Equine platelet lysate (ePL) has shown to improve culture conditions for equine mesenchymal stem cells (MSCs), without altering proliferation rates, phenotype or function, and resulting in superior cell viability.[72] Moreover, concentration of growth factors such as platelet derived growth factor-BB from pooled ePL is higher when compared to HS.[77] To our

knowledge, there are no reports on the effects of ePL as a supplement of EGM to study *ex vivo* angiogenesis in horses.

The mouse aortic ring assay is a common *ex vivo* model to study vascular formation, being suitable in both anti- and pro-angiogenic studies. Aortic explants have the capacity to form branching microvessels when embedded in extracellular matrix. Furthermore, it is relatively low cost with the benefit of no interventions performed in live subjects.[44] Although there is inherent variability in the angiogenic response of rings from the same animal, this system can be adapted to different experimental conditions, and the endothelium of the explants behaves similarly to endothelial cells *in vivo*.[43, 45]

This assay has been used in other species such as chick embryos, dogs, humans, cows, and a single report of the use in horses.[39, 42] Equine arterial rings were used to evaluate the effects of cortisol on angiogenesis, by adapting the mouse aortic ring to equine facial and laminar arteries.[46] However, there are no reports of a wider use of equine arterial rings to evaluate angiogenesis and how this *ex vivo* model responds to different endothelial growth factors.

The aims of this study were to describe the arterial ring assay for evaluation of *ex vivo* angiogenesis in horses by using equine facial arteries, and to evaluate the effects of various types of growth media on vascular network formation. We hypothesized that equine arterial rings serve as an *ex vivo* model to study angiogenesis in horses, and sprouting angiogenesis will be enhanced when using ePL versus the standard HS supplemented EGM.

4.2 Methods

4.2.1 Animals

For harvesting of platelet-rich plasma (PRP), 1 L of whole blood was collected from each of 6 healthy university-owned horses (American Quarter Horse (n=4), and Thoroughbred (n=2), ages 5-16 years). A complete blood count was performed to determine the baseline platelet concentrations.

For the *ex vivo* angiogenesis assay, tissue containing facial arteries was collected within 5 minutes post-euthanasia from healthy horses euthanized for reasons unrelated to this study. To evaluate the dynamics of sprouting angiogenesis and the effect of growth factors in vascular network formation, facial arteries from 5 horses [American Quarter Horse (n=2), Warmblood (n=1), and Thoroughbred (n=2), ages 2-20 years] were collected post-euthanasia.

To evaluate the angiogenic effects of ePL, facial arteries from 6 additional horses [American Quarter Horse (n=4), and Thoroughbred (n=2), ages 4-15] were used. Euthanasia was performed by intravenous injection of pentobarbital sodium and phenytoin sodium solution (Euthasol®) at a dose of (78 mg/kg). Use of animals was approved and monitored by the Auburn University Institutional Animal Care and Use Committee (protocols 2020-3745, 2020-3809).

4.2.2 Facial artery dissection and arterial ring preparation

Tissues were maintained at 4°C in serum-free endothelial basal media (EBM). A branch of the transverse facial artery of 1 mm diameter, was dissected and cleaned of surrounding

adipose tissue. The lumen of the arteries was rinsed to remove blood clots with cold (4°C) EBM using a 25 gauge needle attached to a 1 mL syringe. Arteries were cut into 1 mm rings using a scalpel blade and placed in cold (4°C) EBM. Each artery yielded between 15 to 20 rings (Appendix B).

Matrigel® (BD Biosciences, Bedford, MA, USA) was prepared as per manufacturer's instructions. Briefly, it was thawed overnight at 4°C, and 40 μL were added to each well of a chilled (-20°C) 96-well plate, and allowed to polymerize for 15 minutes in an incubator at 37°C, 5% CO_2. Each ring was dried using task wipers and placed over the Matrigel®, with the lumen parallel to the gel surface. An additional 40 μL of Matrigel® were placed on top of the ring so it was fully covered. Matrigel® was allowed to polymerize before exposing the rings to the study conditions.

4.2.3 Preparation of equine platelet lysate

Platelet-rich plasma was obtained based on a simple tube centrifugation protocol.[157] Briefly, 1 L of blood was collected aseptically from the jugular vein and into a blood collection bag containing citrate phosphate dextrose adenine anticoagulant (126 mL). Blood was centrifuged at 345 xg, 4°C for 10 minutes, and the plasma further centrifuged at 615 xg 4°C for 5 minutes. Supernatant was centrifuged at 1,160 xg 4°C for 5 minutes. The platelet-poor plasma (PPP) was decanted and saved for future use. The remaining PRP pellet from the 6 horses was pooled, yielding a mean platelet concentration of $2{,}758 \times 10^3$/mL. PRP was stored at -80°C.

To produce the lysate, platelets were fractured using 3 freeze-thaw cycles. Samples frozen at -80°C were thawed at 37°C, and 2 more cycles were completed. Confirmation of cell lysis and absence of white blood cell (WBC) contamination was done by measuring the platelet concentration ($1x10^3$/mL) and WBC count ($0x10^3$/mL). Based on a mean baseline platelet concentration of $120x10^3$/mL, and the mean PRP platelet concentration between the 6 horses, a mean of 24-fold increase was obtained. The ePL was diluted using pooled PPP to a concentration of 10-fold from the baseline platelet concentration and centrifuged once at 4,800 xg 4°C for 60 minutes, followed by 2 centrifugations of 30 minutes each at 4,800 xg room temperature. Supernatant was collected and stored at -80°C for future use. The ePL at 10-fold concentration was diluted by adding pooled PPP to produce 2-, and 5-fold increases from baseline platelet concentrations. Samples were processed using sterile technique within a biosafety cabinet.

4.2.4 Preparation of endothelial growth media

Endothelial growth media (EGM-2 with Bullet Kit, Lonza, Visp, Switzerland), free of FBS was prepared as per manufacturer's instructions by mixing the growth factor-free EBM with the Bullet Kit (the Bullet Kit contains human growth factors). For the different conditions, 10% of either HS or ePL at 2-fold, 5-fold and 10-fold increases from baseline platelet concentrations were added to the EGM, and filtered to remove solids, bacteria, or debris using a 0.45 μm cellulose acetate membrane filter.

4.2.5 Dynamics of sprouting angiogenesis

Five rings from each of 3 horses (n=15) were exposed to EGM with 10% HS (EGM+HS, standard equine endothelial cell growth media). Rings were analyzed and photographed daily using an inverted phase contrast microscope at a 20x magnification to evaluate time of appearance of the first sprout (FS), matrix lysis (ML) and vascular regression (VR). FS was defined as the first vessel-like protrusion observed. ML corresponded to the evidence of Matrigel® degradation, seen as a halo on the periphery of the ring. VR was observed as the breakage of the vascular network occurring at the periphery. Arterial rings were evaluated daily, and the time (day) of appearance of the FS, VR and ML were compared between horses.

4.2.6 Effect of growth factors in angiogenesis

Fifteen rings from each of 2 horses were embedded in Matrigel® as previously described. From each horse, 3 rings were randomly assigned to each condition (n=6): a) EGM+HS (endothelial cell growth media containing human growth factors and equine serum, positive control), b) EBM (free of growth factors and serum), c) EBM+human vascular endothelial growth factor (hVEGF), d) EBM+HS, or e) EGM+EDTA (negative control). Media was replaced every 48 hours until analysis at day 7. Vascular network area (VNA) was determined using Fiji software and represented the area in μm^2 contained between the perimeter of the vascular network and the arterial ring. The maximum network growth (MNG) in μm was calculated from the average of the 8 maximum lengths measured from the center of the ring to

the furthest angiogenic sprout. VNA and MNG were compared among groups at day 7. Day 7 was chosen based on results from the previous phase.

4.2.7 Effect of equine platelet lysate on angiogenesis

Arterial rings from the facial artery were dissected and embedded in Matrigel® as previously described. Rings from 6 horses (20-30 rings per horse) were supplemented with EBM (free of growth factors and serum) for 6 days. For inclusion in the study, rings should have evidence of sprouting angiogenesis after culture with EBM (days -6 to 0). Out of 174 rings, 111 met the inclusion criteria. Rings were randomly selected and assigned by triplicate to each of the groups: EGM-10xePL (EGM+10% 10-fold ePL) (n=18), EGM-5xePL (EGM+10% 5-fold ePL) (n=18), EGM-2xePL (EGM+10% 2-fold ePL) (n=18), EGM-HS (EGM+10% HS) (n=18), EGM-PPP (EGM+10% PPP) (n=9), EBM-PPP (EBM+10%PPP) (n=15), and EBM (n=15). Once sprouting was established (day 0), EBM was removed and replaced with 200 μL of each of the study growth medias. Photomicrographs at a 20x magnification were obtained at baseline (day 0), days 1, 2 and 3 (Figure 10).

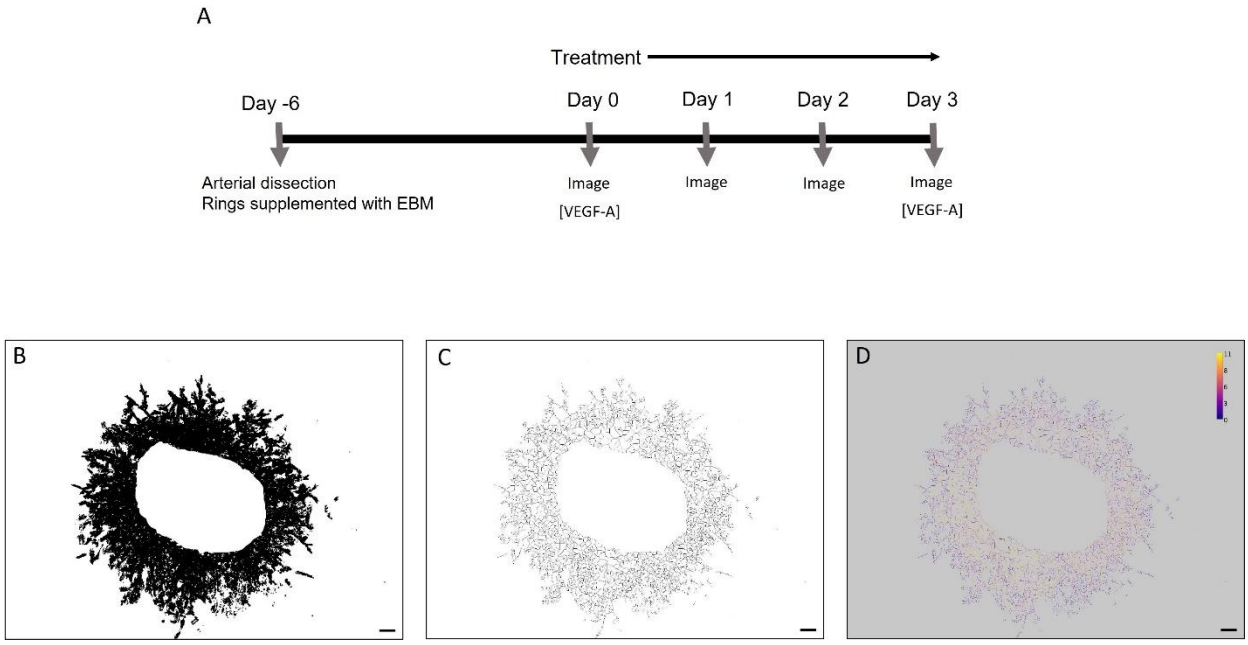


Figure 10. Analysis of the effects of equine platelet lysate on angiogenesis. A) Diagram showing the study timeline. B) For determination of the vascular network area, the background was removed and a binary image created to quantify the area in black. C) The binary image was converted into a single-pixel skeleton, thus allowing for pixel count of branches and determination of vascular density. D) The Strahler analysis of the previous figure assigned different colors to each itineration of the vascular tree in order to count branches at each level. Bar = 200 µm.

4.2.8 Equine VEGF-A concentrations

Supernatants from arterial ring explants (n=12) supplemented with EGM-10xePL, EGM-5xePL, EGM-2xePL, EGM-HS, EBM-PPP and EBM, were collected on days 0 (following 6 days of culture in EBM) and at day 3 for measurement of VEGF-A concentrations by equine-specific ELISA. VEGF-A concentrations were also determined in the different growth medias before being used and defined as baseline. Samples were stored at -80°C until analysis.

ELISA was performed as per manufacturer's instructions (Equine VEGF-A, Kingfisher Biotech, Inc.). Capture antibody (2.5 μg/mL) was prepared by diluting the anti-equine VEGF-A polyclonal antibody (1 mg/mL) in phosphate buffer solution, and 100 μL added to each well of a 96-well plate. The plate was incubated for 12-24 hours, followed by addition of 100 μL of 4% BSA as the blocking buffer and incubated at room temperature for 1-3 hours. Recombinant equine VEGF-A was added as the standard. Samples were added to the wells and incubated for 1 hour at room temperature. Detection antibody (biotinylated anti-equine VEGF-A polyclonal antibody) at a concentration of 0.2 μg/mL was added to the wells after washing (0.05% Tween-20). Following another wash, streptavidin-HRP was added and incubated for 30 minutes. Tetramethylbenzidine substrate solution was added to the wells and developed in the dark for 30 minutes. Immediately after, the stop solution was added and absorbance measured at a wavelength of 450 nm using a plate reader (SpectraMax iD5®).

4.2.9 Image analysis

Image processing and analysis was further refined, and photomicrographs modified using Fiji image processing software. Images were converted into 8-bit, and the low intensity background removed by adjusting the gamma feature to values between 1-1.5. A bandpass filter between 2 and 50 pixels was applied to filter out large and small structures. Following this, the edges were highlighted by using a variance filter. To reduce background artifacts, a subtraction of 100 was applied.[158]

The image was converted to binary with pixel intensities of 0 for white and 255 for black. A cycle of close-dilate was done to smooth objects and fill in small holes. Finally, any artifact was manually deleted to isolate the vascular tree. To obtain the VNA in μm^2, the threshold was adjusted to 255. The binary image was skeletonized, and using histogram analysis, the number of pixels in black (255) was recorded for the vascular density. Finally, a Strahler analysis (http://fiji.sc/Strahler_Analysis) was applied to obtain the number of branches (Figure 10). A complete sequence of the image modification process is represented in appendix C.

4.2.10 Statistical analysis

Statistical analysis was done using commercial software (SAS 9.5M7 or GraphPad Prism 9.3.1). Normality was assessed by Shapiro-Wilk test and QQ plots. Medians of FS, VR, ML between horses and VNA and MNG between conditions were compared by Kruskal Wallis tests

with Dunn's post hoc tests. Time of appearance of FS, VR and ML was recorded for descriptive statistics.

To compare the effects of EGM supplemented with ePL, HS or PPP, EBM-PPP and EBM, rings exposed to the 3 different concentrations of ePL (EGM-2xePL, EGM-5xePL and EGM-10xePL) were combined into a single group (n=54). Following normalization for baseline, means were compared among groups at days 1, 2 and 3 by two-way repeated measures ANOVA with Tukey's post hoc test. VEGF-A concentrations between rings exposed to the different growth medias was compared by one-way ANOVA with Tukey's post hoc test. The effect of the different concentrations of ePL on vascular network formation and the growth rate between rings exposed to the different growth medias were compared by linear regression. The effect of VEGF-A in the number of branches, density and VNA on day 3 was determine by Pearson correlation. $P<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Assessment of sprouting angiogenesis

All equine arterial rings exposed to EGM+HS were able to produce new blood vessels after 5 days, with the earliest being 3 days (median = 4.47 days). Vascular regression was observed as early as day 9, with a maximum of 13 days (median = 10.73 days). Finally, ML was not observed until day 11, with a maximum of 13 days (median = 12.07 days) (Figure 11). A window between day 5 and 9, based on the maximum for FS and first evidence of VR was determined to be optimum for ring selection in further experiments. Within the different

groups, no statistically significant differences were observed between the 3 horses for FS ($p=0.3934$), VR ($p=0.0607$) or ML ($p=0.2364$).

4.3.2 Effect of growth factors in angiogenesis

Arterial rings exposed to EBM and EGM+EDTA with growth media change every 48 hours did not show any vascular growth. VNA in EGM+HS was significantly larger than EBM ($p=0.0015$) and EGM+EDTA ($p=0.0015$). Significant differences in MNG were observed between EGM+HS when compared to EBM+hVEGF ($p<0.0001$), EBM ($p<0.0001$) and EGM+EDTA ($p<0.0001$). Both EBM+HS and EBM+hVEGF had significantly larger MNG when compared to EBM and EGM+EDTA ($p<0.0001$). A trend towards higher MNG was observed for the groups containing HS (EGM+HS and EBM+HS) when compared to EBM+hVEGF (Figure 12).

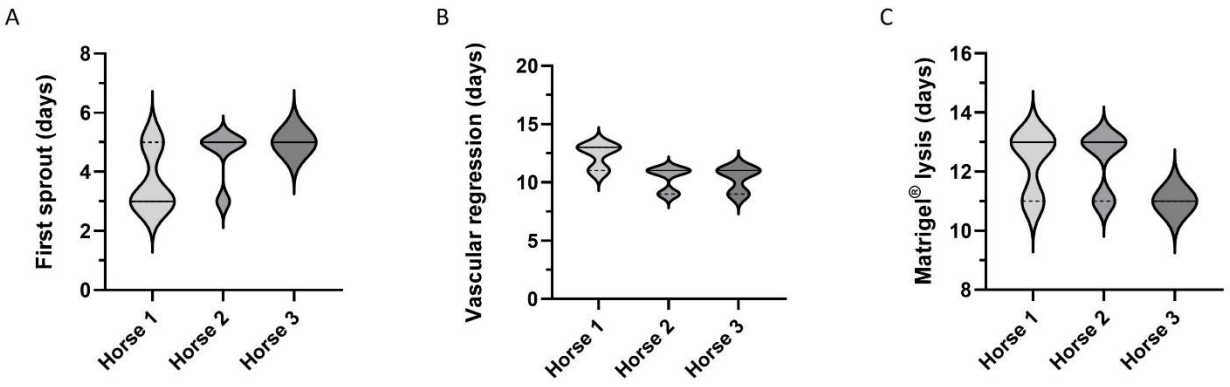


Figure 11. Dynamics of sprouting angiogenesis. Violin plots representing the distribution of dynamics of sprouting angiogenesis for appearance of A) first sprout, B) vascular regression and c) Matrigel® lysis in days from 15 arterial rings of 3 horses (n=5) supplemented with EGM-HS (endothelial growth media + horse serum). The median is represented by the solid line, and the 25th and 75th percentiles are represented by the dashed line.

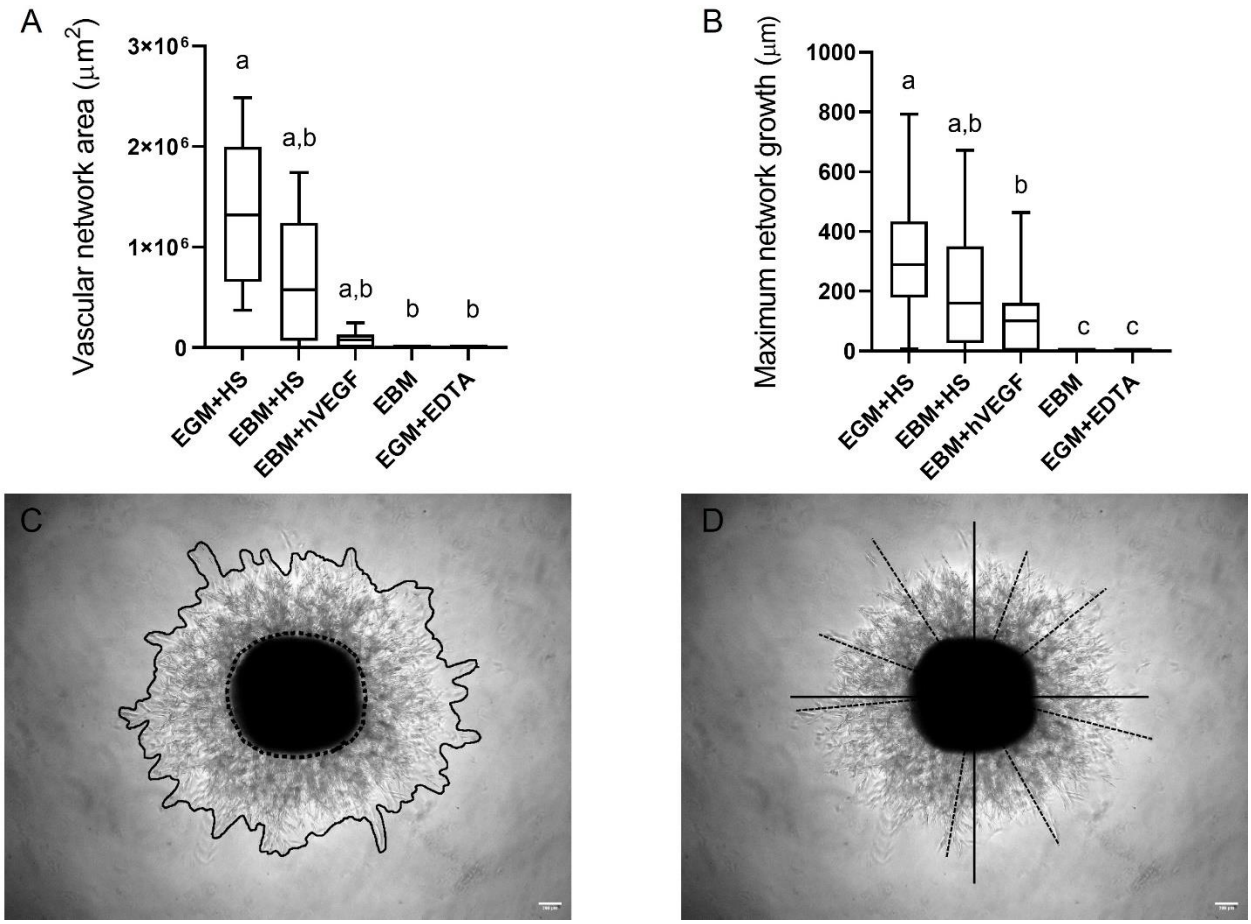
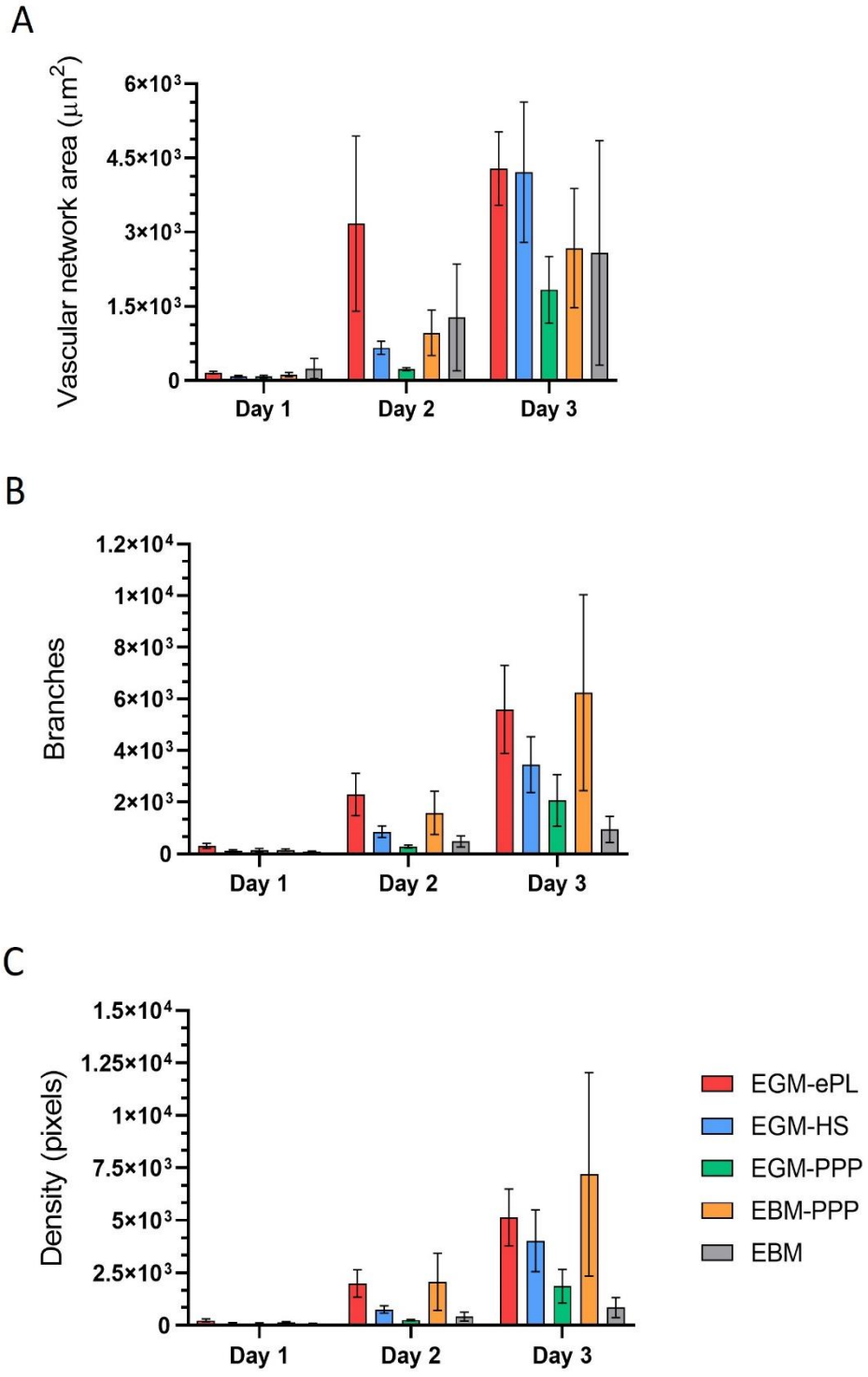


Figure 12. Effect of growth factors on vascular network formation. Side-by-side box plots comparing the (A vascular network area and (B maximum network growth, from the arterial rings after 7 days of exposure to the different growth medias (n=6). C) Vascular network area was the difference of the area inside perimeter of the vascular growth (solid line) and the perimeter of the ring (dotted line). B) Maximum network growth was determined by dividing the arterial ring in quadrants (solid lines). For each quadrant, the 2 maximum growths from the surface of the ring (dotted lines) were recorded. Finally, the 8 maximum growths obtained were averaged. The lower and upper limits of the box represent the 25th and 75th percentiles, median is represented by the horizontal line within the box, and the whiskers delimit the range. Statistically significant differences are present when the letters above each of the boxes differ between groups. Bar = 200 µm.

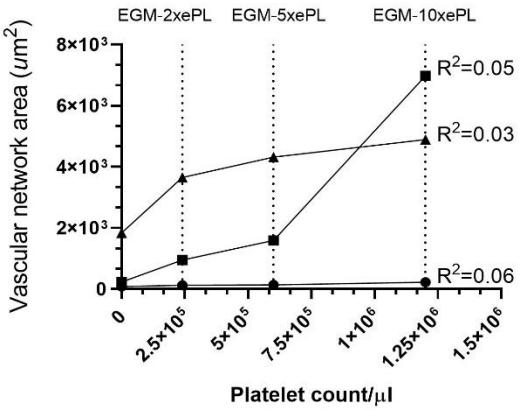
4.3.3 Effect of equine platelet lysate on angiogenesis

After 3 days of exposure to the different conditions, no overall significant differences between treatments were observed at any time point for VNA ($p=0.6271$), number of branches ($p=0.5014$) or density ($p=0.4394$). Some treatments significantly increased the number of branches, VNA and density over time more than others, and a significant effect of time in the different angiogenic parameters was observed ($p<0.0001$) (Figure 13). However, when comparing growth rates between the different treatments, no significant differences were observed for VNA ($p=0.9825$), branches ($p=0.4082$) and density ($p=0.3048$).

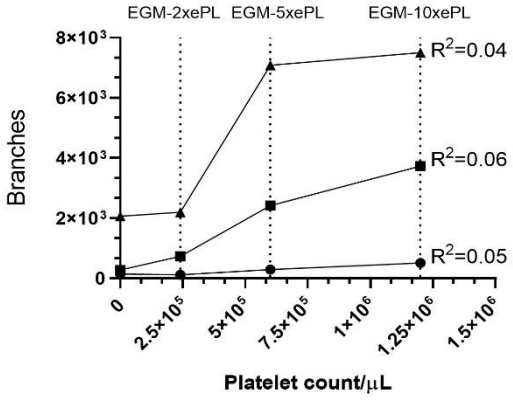
The R^2 values were positive indicating increases in VNA, branches, and density with increasing concentrations of ePL, however no difference was observed between slopes at the different days for VNA ($p=0.1554$), number of branches ($p=0.2923$), or density ($p=0.1964$), and the slopes were not significantly different from 0 by day 3 (Figure 13).


A
Vascular network area (μm²)
6×10³
4.5×10³
3×10³
1.5×10³
0
Day 1
Day 2
Day 3
B
Branches
1.2×10⁴
1×10⁴
8×10³
6×10³
4×10³
2×10³
0
Day 1
Day 2
Day 3
C
Density (pixels)
1.5×10⁴
1.25×10⁴
1×10⁴
7.5×10³
5×10³
2.5×10³
0
Day 1
Day 2
Day 3
EGM-ePL
EGM-HS
EGM-PPP
EBM-PPP
EBM

D


EGM-2xePL
EGM-5xePL
EGM-10xePL
Vascular network area (um2)
8×10³
6×10³
4×10³
2×10³
0
R2=0.05
R2=0.03
R2=0.06
0
2.5×10⁵
5×10⁵
7.5×10⁵
1×10⁶
1.25×10⁶
1.5×10⁶
Platelet count/µl

E


EGM-2xePL
EGM-5xePL
EGM-10xePL
Branches
8×10³
6×10³
4×10³
2×10³
0
R2=0.04
R2=0.06
R2=0.05
0
2.5×10⁵
5×10⁵
7.5×10⁵
1×10⁶
1.25×10⁶
1.5×10⁶
Platelet count/µL

F

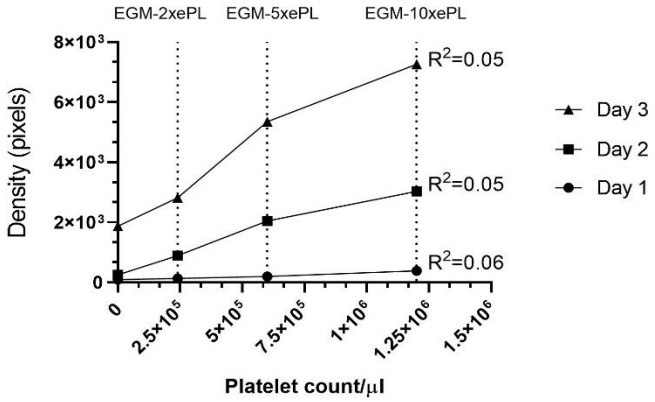

EGM-2xePL
EGM-5xePL
EGM-10xePL
Density (pixels)
8×10³
6×10³
4×10³
2×10³
0
R2=0.05
R2=0.05
R2=0.06
0
2.5×10⁵
5×10⁵
7.5×10⁵
1×10⁶
1.25×10⁶
1.5×10⁶
Platelet count/µl
Day 3
Day 2
Day 1

Figure 13. Effects of equine platelet lysate on vascular network formation. A) vascular network area, B) number of branches, and C) vascular density, based on the number of pixels, for rings exposed to the different conditions after 3 days with no growth media change. No overall statistically significant differences were observed for any of the treatment. Mean ± standard error of the mean is represented in each bar. The effect of increasing concentrations of ePL on vascular growth at each time point are represented by the means for D) vascular network area, E) number of branches and F) density. The concentration of ePL (x axis) corresponds to the fold increase in platelets from the mean baseline platelet concentration, with 2-fold, 5-fold and 10-fold being 2x, 5x and 10x respectively and indicated by the vertical dashed lines. EGM-PPP was used as the negative control (x=0).

4.3.4 Equine VEGF-A concentrations

On day 3, VEGF-A concentration was significantly different between groups (p=0.0182), with a higher trend for the rings exposed to EGM containing ePL at any concentration and HS (Figure 14).

As VEGF-A concentration increased, particularly for the groups containing ePL and HS, there was a positive effect on VNA (p=0.0243) (Figure 14). Descriptive statistics for concentrations of VEGF-A are summarized in table 4.

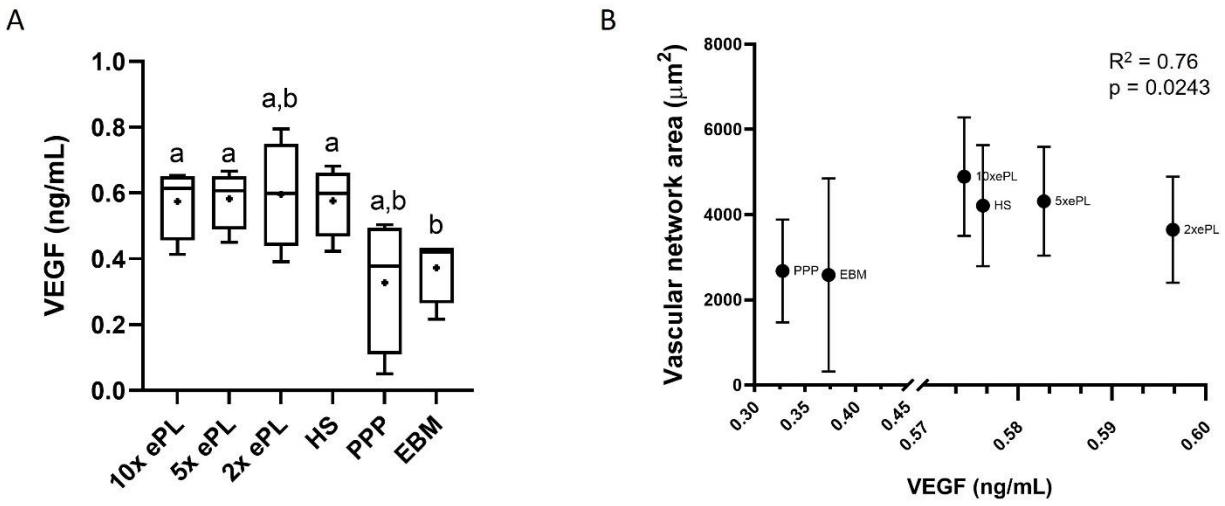


Figure 14. Concentrations of vascular endothelial growth factor A (VEGF-A) in supernatants of media of arterial rings. A) Side-by-side box plots comparing VEGF-A concentrations in supernatants after 3 days of exposure of the arterial rings to the different growth medias (n=12). The lower and upper limits of the box represent the 25th and 75th percentiles, median is represented by the horizontal line within the box, and the whiskers delimit the range. The dot represents the mean. Statistically significant differences are present when the letters above each of the boxes differ between groups. B) Mean ± SEM of the change in vascular network area in relation to VEGF-A concentrations. Note the different scale for the two segments of the x axis.

Condition	Baseline	Day 3	
	Mean ± SD (ng/mL)	Mean ± SD (ng/mL)	Range (ng/mL)
10x ePL	0.074 ± 0.006*	0.574 ± 0.111	0.414 – 0.653
5x ePL	0.074 ± 0.006*	0.583 ± 0.092	0.450 – 0.666
2x ePL	0.251 ± 0.350	0.597 ± 0.165	0.391 – 0.795
HS	0.096 ± 0.040	0.576 ± 0.109	0.424 – 0.682
PPP	0.074 ± 0.006*	0.328 ± 0.207	0.050 – 0.504
EBM	0.074 ± 0.006*	0.373 ± 0.105	0.217 – 0.434

Table 4. Vascular endothelial growth factor A concentrations in the supernatants of arterial rings. Mean ± SD of VEGF-A supernatant concentrations on day 0 (baseline) and day 3 of arterial rings exposed to different conditions. The asterisk (*) indicates that concentrations were below the assay's limit of detection of 0.074 ng/mL.

4.4 Discussion

Here it was described the *ex vivo* angiogenic response of equine arteries when exposed to EGM supplemented with different sources of growth factors by the use of an arterial ring assay. This *ex vivo* model using equine arteries has been described in a report that studied the effect of glucocorticoids on angiogenesis, however FBS was used as a supplement to the EGM.[46] Our results showed that equine arterial rings supplemented with EGM+HS every 48 hours are able to sprout new vessels. Furthermore, equine arterial rings supported sprouting angiogenesis in serum-free media, allowing for later testing of different biological products as candidates for regenerative angiogenesis. An important finding during the initial phase was the fact that equine arterial rings can respond differently depending on the presence of growth factors, and greater vascular growth was observed in the group exposed to EBM-HS compared to EBM alone, suggesting that equine-specific growth factors stimulate the angiogenic response of equine arteries. Although not statistically significant, this could be supported by the fact that rings exposed to EBM+HS had larger VNA and MNG when compared to rings exposed to human VEGF (EBM+hVEGF).

Due to the variability in angiogenic response between rings, preselecting samples with similar angiogenic responses after sprouting has started, similar to studies in rat models, is important.[159] The determination of parameters such as the appearance of FS was critical as a starting point for determining the best time for ring selection. These results showed that the appearance of the FS in horses, around day 5, was similar to reports using rats, where new blood vessels were present between days 3-5 of culture.[47, 159] When using Matrigel®, we observed that neovessels arising from equine arteries will undergo regression as early as day 9,

thus analysis between days 5 and 9 of culture was optimal. Although a different type of matrix was used, the dynamics observed with equine arterial rings explants is similar to reports using rat aortas in a collagen matrix, where the neovessel growth phase starts after 2 to 3 days of culture and continues until regression between day 7 to 10. This regression consists of a process of fragmentation, disintegration and retraction, and is often associated with degradation of the basement membrane.[43]

This study demonstrated that sprouting angiogenesis in the equine arterial ring assay is dependent on the growth media used. Using this model with equine arteries allowed for both stimulation of angiogenesis through the addition of growth factors, as well as inhibition by the addition of EDTA. Thus, this assay can be used to test both promotion of equine angiogenesis as well as regression. As expected by using equine tissues, supplementation of EBM with equine-specific growth factors contained in HS resulted in better vascular network area than EBM containing human VEGF-A, although this difference was not statistically significant. Moreover, the lack of difference in VNA and MNG between rings exposed to EGM+HS and EBM+HS denotes the major influence of HS in angiogenesis, independently of other factors contained in the company provided growth factor cocktail with of EGM. Equine serum contains angiogenic growth factors such as epidermal growth factor, transforming growth factor-β, insulin-like growth factors and most importantly VEGF-A. VEGF-A is highly implicated in the recruitment of tip cells and initiation of sprouting angiogenesis, thus providing equine VEGF-A through equine-derived blood products will stimulate VEGF receptor 2 with high specificity.[21, 160] This is important since this assay can be implemented to study the allogenic use of different biological products for regenerative medicine.

The use of alternative allogenic sources of vascular growth factors was evaluated, and ePL was demonstrated to be a good alternative to provide equine-specific factors to induce sprouting angiogenesis. The treatment type and time that the rings were exposed to the different conditions had an effect in vascular network formation. Furthermore, using ePL seemed to be comparable to the use of HS to support vascular growth. Interestingly, the EBM-PPP group also trended towards a higher number of branches, VNA and density, which was an unexpected result since PPP is considered to have significantly less VEGF concentration when compared to PRP or platelet lysate.[161] Even though there are no reports of concentrations of growth factors in equine PPP, it has been observed that mouse PPP enhances angiogenesis when used in mouse wounds compared to the use of PRP, evidenced by a higher blood vessel density and expression of VEGFR-2.[162] Moreover, human umbilical cord endothelial cells cultured for 48 hours in the presence of human PPP had greater expression of the endothelial cell marker CD34 and VEGFR-2 compared to cells exposed to PRP.[162] The effects of PPP in our study could be explained by the fact that PRP at high concentrations can elicit an inhibitory and cytotoxic effect. Moreover, PPP is also metabolically active and may also stimulate tissue regeneration by its bioactive molecules such as platelet-derived growth factor and insulin growth factor-1.[163] Future studies assessing the cytotoxic effects of blood products in equine endothelial cells are warranted to determine the ideal concentrations to use in cell culture. Results from this study demonstrated a trend toward an increase in vascular growth with higher ePL concentrations. However, a study evaluating equine MSCs, observed a dose-dependent response to addition of ePL up to a concentration of 30%, with further increases resulting in inhibition of cell proliferation.[164] Therefore, further studies with a higher sample size

evaluating a wider range of ePL concentrations are required to determine the maximum concentration that will support vascular growth without the potential to inhibit angiogenesis.

There is limited information regarding the concentration and composition of growth factors in HS and ePL, however it has been reported that concentrations in ePL are higher than previously reported in HS, specifically for TGF-β1 and PDGF-BB.[76, 77] In the current study it was obtained a lower concentration of equine VEGF-A in ePL than previously reported, however a different assay was used and a smaller number of animals.[77] Even though large variations in VEGF-A concentrations were not observed, a previous report found the opposite, with large ranges observed between horses, hence of the importance of using pooled ePL.[77] This study is consistent with others, demonstrating that ePL may be an alternative source of growth factors for cell culture, improving the angiogenic signaling pathway towards an endothelial phenotype.

Other growth factors, beside VEGF-A, contained in ePL, PPP or HS were not measured in the present study, therefore the VEGF-A concentrations observed on day 3 could be influenced by other factors upregulating the release of VEGF-A from the explants. Such is the case of PDGF-BB contained in biological products, which can upregulate the release of VEGF-A by the endothelial cells.[165] Even though the results showed a positive correlation between VEGF-A concentrations and VNA, no group was exposed to growth media depleted of VEGF-A, therefore other growth factors contained in the biological products could have enhanced vascular network formation. This study also showed evidence of the endogenous production of VEGF-A by equine arterial rings, and this might explain the fact that sprouting occurs in rings exposed with EBM for 6 days when growth media is not changed. This has been explained in rat arteries,

where the release of growth factors and cytokines is responsible for paracrine stimulation of sprouting angiogenesis.[159] This finding is of importance, since equine arterial rings can be used to study the effects of different growth factors, without the interference from other factors used to start sprouting. Interestingly, for the first phase of this study, rings exposed to EBM that had media change every 48 hours did not show sprouting angiogenesis, which could be due to the removal of endogenous growth factors from the old growth media.

Different approaches have been described to analyze angiogenesis using this *ex vivo* model. Historically manual sprout count has been done, however due to the exponential growth leading to a high number of branches and the 3 dimensional nature of the set-up, visual real-time count is complicated.[166] Different software have capabilities for counting blood vessels, however image modifications are required to reduce background artifacts.[22, 42] Here a protocol used by Rohban *et al*., 2013 was modified in order to remove the background and isolate the vascular tree as a binary image, which proved to be an effective approach to have objective measurements for VNA and density.[166] Furthermore, it was demonstrated the utility of the Strahler analysis, originally developed for neural cells, for counting terminal branches of a vascular tree.

Some limitations of this *ex vivo* assay using rats or mice include the variability in angiogenic responses among different animals or rings, as well as differences in ring dimensions.[39] Variability among rings in this study is illustrated by the wide error bars on figure 13. This is likely due to the use of animals within a wide range of ages (2-20 years old), and the high genetic diversity of horses versus similar ring models in mice or rats. It has been determined that older mice and rats produce fewer vessels compared to younger.[45, 167]

With high variability, achieving statistical power was a limitation of this study. This study aimed for an overall medium effect size of 0.5 (Cohen's d), which was achieved based on the data. With a medium effect size and no overall statistical difference observed, the existence of a true direct effect of ePL on vascular growth should be interpreted with caution. This data only achieved a Cohen's d effect size of 0.6 when comparing EBM alone with the ePL groups, where a larger difference was expected. Therefore, increasing the sample size may increase the magnitude, or at least the precision, of the effect size, and statistically significant effects of ePL on vascular growth may be detected. Another limitation was the use of phase contrast microscopy alone with no immunostaining for endothelial cells, which could have aid in discriminating endothelial cells from other cells at the initial stages of angiogenesis.[130] However, the exponential growth observed in the microvessel-like structure, and the long sprouts were most likely consistent with sprouting angiogenesis.

Matrigel® is of xenogenic origin when used with equine arteries, and will contain mouse growth factors, making difficult the isolation of equine-specific growth factors as players in angiogenesis. To account for this, all arterial rings were grown in EBM (no growth factors) prior to exposure to the study conditions; furthermore, the data was corrected for baseline to reduce the intrinsic effects of Matrigel® in vessel growth. Despite these limitations, this study is a starting point for evaluation of ePL on vascular growth and as a potential source of growth factors in cell expansion.

It was concluded that the method established for the mouse aortic ring assay could be used with equine arteries to study the effect of allogenic sources of growth factors. This method provides large sample size, high reproducibility and low cost, and it is an option to

consider before moving into *in vivo* experiments.[42] The results show that there is variability in responses of rings, that are likely inherent to the horse, despite selecting rings that demonstrate sprouting activity for experimental conditions. Nonetheless, it was demonstrated that supplementation with PPP, ePL, and HS are able to support sprouting angiogenesis in this model. The results obtained are important for future selection of the most appropriate culture conditions for equine ECFCs, and it serves as a starting point for studies using coculture of progenitor cells with an *ex vivo* model.

Chapter 5 – Addition of a nitric oxide donor does not affect equine endothelial colony-forming cells phenotype and function

5.1 Introduction

Endothelial cell (EC) phenotype is influenced by many different factors, and since these cells are exposed to a variety of microenvironments, the signals from one organ or determined region is enough to generate differences in phenotype across the vascular tree. However, when grown in culture, the lack of exposure to these extracellular signals from the microenvironment can trigger phenotypic drift, mostly by paracrine signaling from other cell types.[168, 169] Equine endothelial colony-forming cells (ECFCs) have been successfully sorted based on expression of the endothelial cell (EC) surface marker cluster of differentiation (CD) 31 and uptake of acetylated low density lipoprotein (Ac-LDL). However, once expanded in culture, this phenotype is lost, with CD31-negative cells predominating, thus there is a need to improve isolation and culture conditions of equine ECFCs in order to stimulate the signaling pathways towards an endothelial phenotype.

The introduction of vascular signaling factors and equine-specific growth factors to the cell culture conditions, has the potential to further preserve the EC phenotype of cultured ECFCs. Nitric oxide (NO) is an endothelium-derived relaxing factor with functions of vasodilation, vascular permeability, and antithrombosis. Furthermore, it has cardiovascular homeostatic and vasoprotective properties, and it may play an important role in cardiovascular regenerative processes.[86, 87] Endothelial nitric oxide synthase (eNOS) is the NOS isoform

responsible for the conversion of L-arginine to L-citrulline to produce NO, and it is highly expressed in ECs.[19, 86] This gaseous radical is continuously synthesized due to its short half-life and acts as a chemical messenger primarily in the vascular and immune systems.[88]

Signaling via receptors, such as vascular endothelial growth factor receptor-2 (VEGFR-2), and interactions with other proteins regulate eNOS activity and NO production.[82] eNOS is highly expressed in vascular ECs, including endothelial progenitor cells (EPCs), and it is believed that the main pathway for NO as a key player in EPC mobilization and function is by interaction and stimulation of the VEGFR-2.[19, 87] Although it is well known that VEGF-A upregulates the expression of eNOS leading to the release of NO, this cascade of events may also occur in the opposite direction. In rat and human vascular smooth muscle cells, the release of VEGF into the culture media and VEGF mRNA expression were significantly higher in the presence of NO donors 3-morpholinosydnonimine and S-nitroso-N-acetylpenicillamine (SNAP). Furthermore, this upregulation was reversed by the NOS inhibitor L-N^G-Nitro arginine methyl ester (L-NAME).[83] Similar results have been observed with increasing protein expression of VEGF following SNAP treatment of cultured rat cardiomyocytes.[89] Additionally, in cultured human umbilical vein ECs, greater *in vitro* angiogenesis was observed when a NO donor was added to the endothelial growth media (EGM), when compared to non-treated ECs. In this case, the NO donor was observed to activate the angiogenic signaling pathway phosphoinositide 3-kinase/protein kinase B through non-hypoxic hypoxia-inducible factor-1α (HIF-1α).[89] Moreover, NO plays a role in the different key processes of angiogenesis, including dissolution of matrix, EC proliferation and migration, and the final formation of tubules.[83]

It has been observed that in eNOS-depleted mice, there is a significant reduction in EPC function, and EPCs pretreated with an eNOS enhancer improve neovascularization in an ischemic model.[87] Nitric oxide is a free radical with a short half-life, and it is a potent signaling molecule at low concentrations; however, it will have a cytotoxic effect at high concentrations.[90, 91] In physiological conditions, concentrations of NO as low as 10 nM are produced by eNOS, acting both as vasodilator and inhibitor of platelet aggregation. During inflammation, excessive amounts of NO, (above 1 mM), can be produced, especially by the inducible NOS isoform, which can modulate the activity of inflammatory mediators.[88] The various effects of NO will depend on the site of synthesis, concentrations released, and the type of target tissue.

Nitric oxide donors to deliver supplementary NO have become an attractive therapeutic option in the treatment of cardiovascular diseases, and they can release NO by different mechanisms, including spontaneous, chemical catalysis or enzymatic reaction. S-nitroso cysteine (CysNO), a NO donor from the S-nitrosothiols class, will release NO spontaneously through thermal or photochemical self-decomposition.[88]

Previous studies used the addition of NO to improve cardiomyocyte differentiation in suspension of both mouse and human cells, providing a more inexpensive method for differentiation in a large-scale cell culture.[92, 93] Regarding the effects of NO on ECs, NO has been shown to increased EC proliferation after carotid balloon injury when delivered by a NO-containing hydrogel.[94] The effect of eNOS has been observed to favor ECFCs, where its expression and *in vitro* tubule formation is greater when compared to early EPCs. [13, 19] Moreover, when adding a NO donor to L-NAME treated or eNOS deficient mice, an increase in

bone marrow ECFC mobilization was observed; however, no effect on recruitment of hematopoietic stem cells was observed.[95]

Due to the function and location of ECs, they are in direct contact with blood flow, creating a wall shear stress. When ECs are exposed to shear forces, eNOS will be quickly upregulated, and changes in flow pattern, either in direction or magnitude, can decrease the activity of eNOS, leading to prothrombic and proinflammatory states.[84] When challenging human umbilical vein ECs (HUVEC) to different shear stress magnitudes, the expression of CD31 was significantly decreased in cells under static culture when compared to dynamic. Similarly, exposure to physiological shear magnitudes promoted cell viability and resulted in higher expression of eNOS.[84] Dynamic cultures often require the use of specialized equipment that can be costly or difficult to access; therefore, the addition of exogenous NO could be efficacious in mimicking dynamic cell culture conditions for equine ECFCs. To our knowledge, there are no studies assessing the effect of NO donors in culture of equine ECFCs.

Due to the low number of circulating ECFCs, expansion while preserving the phenotype following isolation is key for future therapeutic use. The use of NO donors, CysNO, could promote ECFCs migration and proliferation, as well as preserving the EC phenotype. However, further studies using equine ECFCs are warranted to determine the ideal concentration of NO donor that will promote signaling pathways without having a cytotoxic effect.

The objectives of this study are to determine the effects of NO in ECFC proliferation, viability, and function *in vitro* when supplementing cultured equine ECFCs at different passages with the NO donor CysNO. Additionally, we aim to evaluate the changes in expression of the EC

marker CD31 in equine ECFCs exposed to NO. We hypothesized that the addition of CysNO to the EGM will promote the angiogenic pathway in ECFC, resulting in cells with more expression of the endothelial marker CD31 and greater tubule formation *in vitro* when compared to ECFCs cultured in EGM alone.

5.2 Methods

5.2.1 Cells

Cryopreserved equine ECFCs from 3 different horses (American Quarter Horse (n=3), ages 5-8 years), and one line of equine carotid-derived ECs, all at passage 2, were seeded into collagen coated culture flasks at a cell density of 12,000 cells/cm^2. Equine ECFCs and ECs had been previously characterized by uptake of Ac-LDL and microtubule formation *in vitro*. Equine ECFCs and EC were used from passages 2 to 6, and were maintained at standard culture conditions of 5% CO_2 and 37°C.

5.2.2 CD31 expression and cell viability analysis

CD31 mouse monoclonal antibody (Anti-CD31 antibody JC/70A Abcam) was tested in cryopreserved equine carotid ECs to confirm reactivity. Equine ECFCs and ECs were thawed and expanded in a collagen-coated flask as previously described. Once 80% confluent, cells were enzymatically dissociated with TrypLE™, centrifuged and resuspended in 10% normal goat serum in phosphate buffer solution (PBS) for 1 hour at room temperature to remove unspecific

protein binding. Cells were washed with PBS, and $3x10^5$ cells transferred to each of the tubes needed for flow cytometry. Prior to adding the primary antibody, cells were treated with Ghost Dye™ (Tonbo biosciences) for live/dead analysis. The primary antibody (CD31) was prepared at a concentration of 2 μg/100 μL, and cells were resuspended in the 100 μL solution for 45 minutes at room temperature. Cells were washed 3 times in PBS and resuspended in 100 μL of diluted secondary antibody (Alexa Fluor™ 188 goat anti-mouse at a 1:400 dilution), and incubated at room temperature for 45 minutes. Cells were washed 3 times in PBS, resuspended in 500 μL 1% BSA, and filtered using a 40 μm cell strainer.

Cells were analyzed in a Beckman™ flow cytometer. A minimum of 10,000 events were analyzed. For analysis, cells were gated to remove debris, doublets and dead cells when live/dead analysis was performed. Therefore, the cell fraction that included single and live cells was used for determination of CD31 expression.

5.2.3 Matrigel® tubule formation

Matrigel® (BD Biosciences, Bedford, MA, USA) was prepared as per manufacturer's instructions. Briefly, it was thawed overnight at 4°C, and 80 μL were added to each well of a chilled (-20°C) 96-well plate and allowed to polymerize for 15 minutes in an incubator at 37°C, 5% CO_2. Following this, 15,000 cells in 200 μL of endothelial growth media (EGM-2 with Bullet Kit, Lonza, Visp, Switzerland) were added on top of the polymerized Matrigel® and incubated under standard cell culture conditions (37°C, 5% CO_2).

5.2.4 Preparation of endothelial growth media

Endothelial growth media (EGM-2 with Bullet Kit, Lonza, Visp, Switzerland), was prepared as per manufacturer's instructions by mixing the growth factor-free EBM with the Bullet Kit and 10% horse serum (HS). Freshly prepared CysNO was added to the EGM+HS to obtain final concentrations of 50, 100 and 200 μM depending on the study performed. Additionally, L-NAME to a final concentration of 1 mM was added when required.

A pilot study was performed evaluating the use of ePL in replacement of the HS for ECFC cell culture, and since no significant benefit was observed from the use of ePL, HS was added to the EGM as traditionally used. A summary of the results of this pilot study are presented in appendix D.

5.2.5 Nitric oxide donor preparation

CysNO at concentrations of 50 μM, 100 μM and 200 μM, was prepared as previously described by Hodge *et al*.[92] An L-cysteine and sodium nitrite solution in N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer (100 mM) was adjusted to a pH of 7.4. The working CysNO solution was prepared fresh prior to each use.[92] The release of NO from the donor in both HEPES and EGM at the 3 different concentrations was determined by the Griess assay as described by Kushwaha *et al*.[170] Following manufacturer's instructions (Griess Reagent System, Promega), a nitrite standard reference curve was prepared ranging from 1.56 μM to 100 μM. For the nitrite measurement, 50 μL of each sample was added to wells of a 96-well plate. Fifty μL of sulfanilamide solution was added to the samples and

standards and allow to incubate at room temperature for 15 mins protected from light. Finally, 50 μL of N-1-naphthylethylenediamine dihydrochloride solution was added to all wells and incubated for 10 minutes at room temperature. Absorbance was measured at 530 nm within 30 minutes using a plate reader (SpectraMax iD5®).[170]

5.2.6 Effect of nitric oxide in equine ECFC function and phenotype

Endothelial colony-forming cells and ECs from one cell line were cultured in EGM containing 50 μM, 100 μM or 200 μM of CysNO for 72 hours to determine cell viability by flow cytometry (FC). Based on the viability results and data from a previous study,[92] a concentration of 100 μM was used for the remaining of the study.

Endothelial colony-forming cells from each of 3 cell lines and equine ECs were randomly assigned to different culture flasks containing EGM (Bullet Kit, Lonza, Visp, Switzerland) + 10% horse serum (HS) with S-nitrosocysteine added at a concentration of 100 μM (EGM-HS/CysNO) or EGM-HS alone. Cell culture media from all groups were replaced every 48 hours. Matrigel® tubule formation *in vitro* (number of segments, branches, meshes, junctions, tube length and mesh area), viability (live/dead), and expression of the EC marker CD31 were evaluated at passages (P) 2, 4 and 6 and compared between the EGM-HS/CysNO and EGM-HS groups within and between timepoints. Additionally, cell seeding density after each subculture, cell number at the time of subculture, and time (hours) between subcultures were recorded and used to determine the number of cell doublings (NCD) in each 24-hour period, the population doubling

time (PDT) in hours, and the cumulative population doubling level (CPDL). Each of these parameters were calculated as follows:

$$NCD = \frac{log2\frac{CH}{CS}}{number\ of\ days}$$

$$PDT = \frac{total\ number\ of\ hours}{NCD}$$

$$PDL = \frac{log10(CH) - log10(CS)}{log10(2)}$$

CH was the number of cells at 80% confluency and CS was the number of cells seeded in the following passage. The calculated PDL at each passage was added to the previous subculture PDL to determine CPDL.

5.2.7 Equine VEGF-A concentrations

For evaluation of the effects of NO in the release of VEGF-A, equine ECs were cultured in EGM+HS containing either: CysNO at 50 μM (EGM-HS/50), 100 μM (EGM-HS/100), 200 μM (EGM-HS/200), or EGM-HS alone. To block the release of endogenous NO, a solution containing NOS inhibitor L-NAME (1mM), was prepared in PBS and added to the EGM of additional cultured EC in the following groups: CysNO at 50 μM + LNAME (EGM-HS/50LN), 100 μM + L-NAME (EGM-HS/100LN), 200 μM + L-NAME (EGM-HS/200LN), or EGM-HS + L-NAME (EGM-HS/LN). Supernatants and cell lysates from cultured ECs at P4 were collected and stored at -80°C until analysis. For cell lysate preparation, the adherent ECs were washed twice with cold

(4°C) PBS. RIPA buffer (Pierce™, Thermo Scientific) was then added at a volume of 400 μL per T25 flask. Following this, the cells were scraped and transferred to a conical tube for a 15-minute incubation on ice. Cells were sonicated three times for 2 seconds at 50% pulse with one minute rest between pulses. Finally, the lysate was centrifuged at 14,000 xg for 15 minutes at 4°C. Supernatants were collected and aliquoted in 100 μL volumes. Samples were frozen at -80°C and stored until analysis. Protein concentration in the lysates was determined using a commercial protein assay (DC Protein Assay Reagents Package, Bio-Rad). A protein standard of BSA in PBS from 0.2 mg/mL to 1.5 mg/mL was prepared, and following manufacturer's instructions, a working reagent mixing 20 μL of reagent S for each 1 mL of reagent A prepared. Five mL of each standard dilution and sample was added to well of a 96-well plate, followed by addition of 25 μL of the prepared reagent A. Finally, 200 μL of reagent B was added in to each well and incubated at room temperature for 15 minutes. Absorbance was read at 750 nm using a plate reader (SpectraMax iD5®). Protein concentrations between 1.4 and 1.6 mg/mL were used for the ELISA analysis.

ELISA was performed as per manufacturer's instructions (Equine VEGF-A, Kingfisher Biotech, Inc.). Capture antibody (2.5 μg/mL) was prepared by diluting the anti-equine VEGF-A polyclonal antibody (1 mg/mL) in PBS, and 100 μL added to each well of a 96-well plate. The plate was incubated for 12-24 hours, followed by addition of 100 μL of 4% BSA as the blocking buffer and incubated at room temperature for 1-3 hours. Recombinant equine VEGF-A was added as the standard. Samples were added to the wells and incubated for 1 hour at room temperature. Detection antibody (biotinylated anti-equine VEGF-A polyclonal antibody) at a concentration of 0.2 μg/mL was added to the wells after washing (0.05% Tween-20). Following

another wash, streptavidin-HRP was added and incubated for 30 minutes. Tetramethylbenzidine substrate solution was added to the wells and developed in the dark for 30 minutes. Immediately after, the stop solution was added, and absorbance measured at a wavelength of 450 nm using a plate reader (SpectraMax iD5®).

5.2.8 Image analysis

Photomicrographs at a 4x magnification were obtained using a phase contrast inverted microscope at 0, 5, and 24 hours for tubule formation. Images were analyzed at 23 hours using the angiogenesis analyzer tool (Fiji image processing software). Number of segments, number of branches, number of meshes, number of junctions, tube length and total mesh area were recorded.

5.2.9 Data analysis

Statistical analysis was done using commercial software (GraphPad Prism 9.3.1). Normality was assessed by Shapiro-Wilk test and QQ plots. For the overall effect of CysNO in cell viability between ECFCs and ECs, the values obtained from the 3 different concentrations were combined and compared by a two-tailed unpaired t-test. The dose effect of the different concentrations of CysNO on cell viability for ECFCs and ECs was determined by linear regression. Means for percentage CD31 expression, percentage live cells, NCD, PDT, and CPDL for ECFCs exposed to 100 μM CysNO (EGM-HS/CysNO) or EGM-HS were compared by 2-way

repeated measures ANOVA with Tukey's post hoc test. Analysis of these parameters between ECs treated with CysNO and EGM-HS alone was done by Mann-Whitney test.

Number of segments, number of branches, number of meshes, number of junctions, tube length, and total mesh area were compared between EGM-HS/CysNO and EGM-HS cells by mixed effect model with Geisser-Greenhouse correction and Tukey's post hoc test for ECFCs and by a 2-way repeated measures ANOVA with Tukey's post hoc test for ECs. $P<0.05$ was considered statistically significant.

5.3 Results

5.3.1 Nitric oxide release and dose response

The release of NO was characterized by Griess assay in both HEPES buffer and EGM-HS immediately after preparation of CysNO. Within the first hour, samples containing CysNO in HEPES in concentrations of 50 μM, 100 μM and 200 μM released 98.18%, 93.53%, and 85% of the NO respectively. Samples containing 50 μM, 100 μM, and 200 μM of CysNO in EGM-HS released 87.16%, 89.55%, and 97.83% of NO respectively in the first hour. The release of nitrite from CysNO is summarized in table 5.

When comparing percentage of dead cells between ECFCs and ECs exposed to the different concentrations of CysNO, ECFCs had significantly more dead cells than ECs when combining all the groups ($P<0.001$). However, the slopes for dose response were not significantly different from zero or between cell types ($P=0.3841$). There was no statistically significant correlation between CysNO doses and percentage of dead cells for ECFCs ($P=0.0849$)

or ECs (P=0.1284). Even though the slopes were not significantly different from zero, an apparent increase in percentage dead cells was observed as the CysNO dose increased, and this was more evident between 100 μM and 200 μM for the ECs (Figure 15). Because of these results, and based on previous reports[92], a 100 μM concentration of CysNO was chosen for the study.

	HEPES		EGM-HS	
Initial concentration (μM)	Nitrite release (μM)	Nitrite release (%)	Nitrite release (μM)	Nitrite release (%)
50	49.09	98.18	43.58	87.16
100	93.53	93.53	89.55	89.55
200	170.64	85	195.65	97.83

Table 5. Percentage of nitrite release from CysNO. Within the first hour, different concentrations of CysNO were added to either HEPES buffer or EGM-HS. The table shows the nitrite release from the NO donor following a Griess assay.

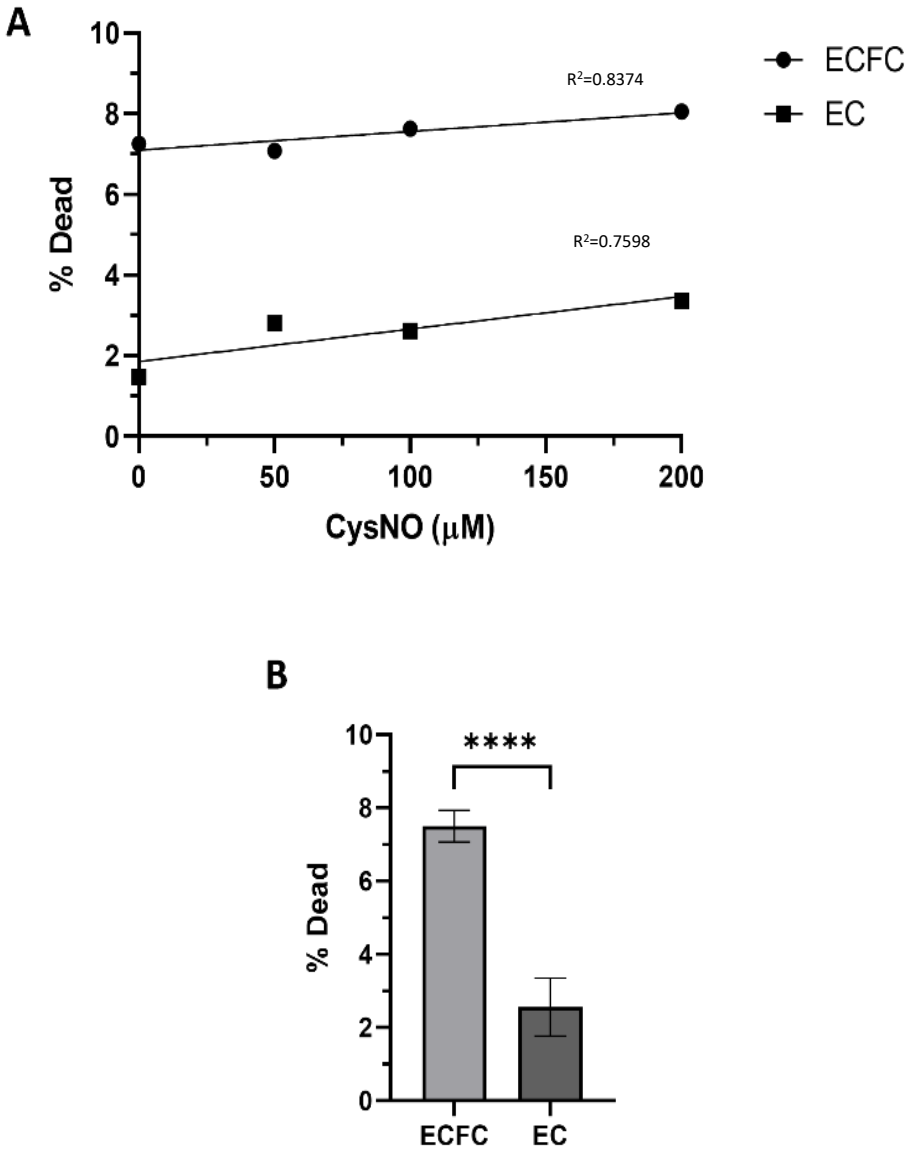


Figure 15. Viability of ECFCs and ECs exposed to different concentrations of CysNO in EGM after 72 hours in culture. A) No statistically significant correlation between CysNO concentrations and cell viability was observed for ECFCs (P=0.0849) or ECs (P=0.1284). B) The overall mean percentage of dead cells was significantly higher in ECFCs when compared to ECs (P<0.001). Data in B is presented as mean ± SD.

5.3.2 Effect of nitric oxide in CD31 expression

The cell passage had a significant effect on CD31 expression (P=0.0049). Specifically, there was a significant decrease in CD31 expression between passages 2 and 6 (P=0.0399), and 4 and 6 (P=0.0231) (Figure 16). No overall statistically significant difference in CD31 expression was observed between ECs treated with CysNO or EGM-HS alone (P>0.9999) (Figure 16). Overall CD31 expression was lower in ECFCs compared to ECs.

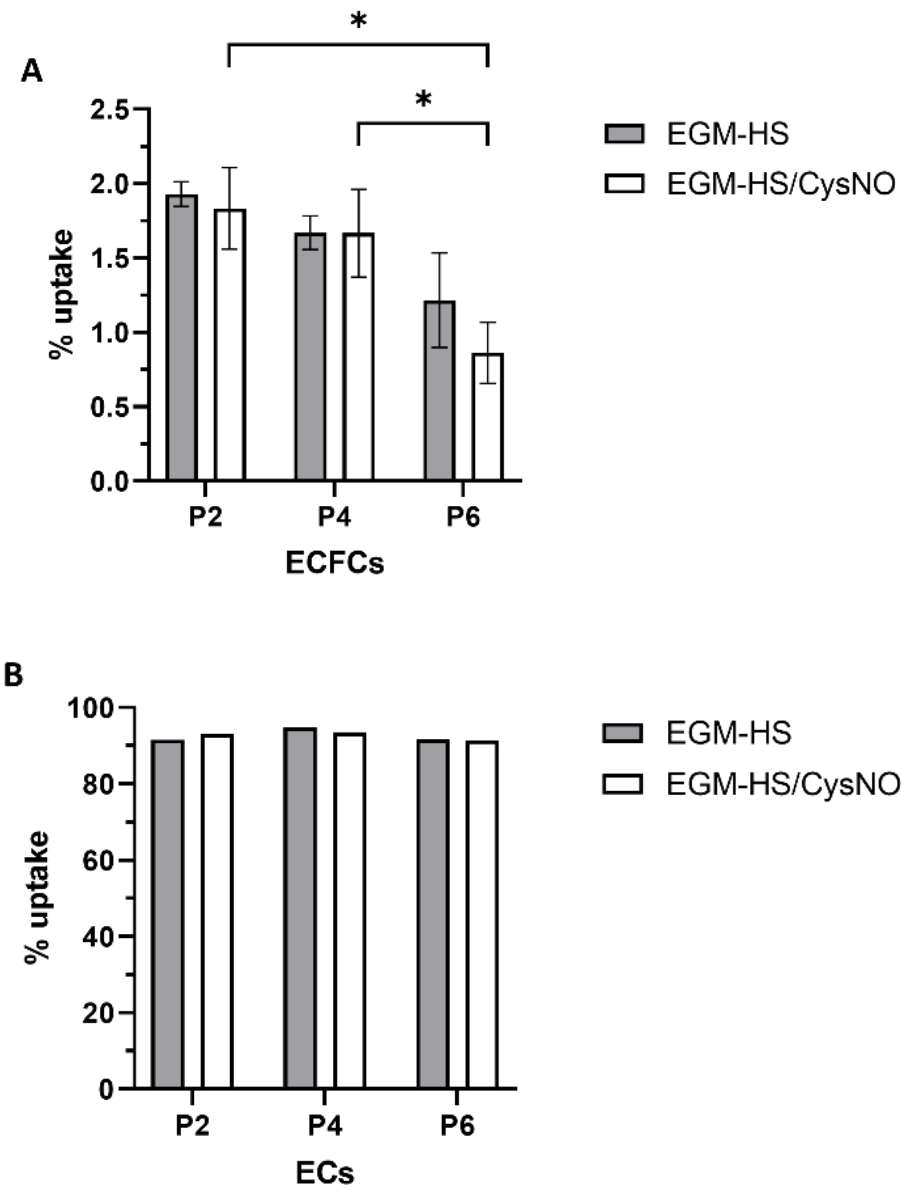


Figure 16. Effect of NO on CD31 expression for equine ECFCs and ECs. Percentage of CD31 expression at 3 different passages for A) ECFCs or B) ECs exposed to either 100 μM of CysNO (EGM-HS/CysNO), or EGM-HS alone. A significant decrease in CD31 expression was observed between ECFCs in the EGM-HS/CysNO group between P2 and P6 (P=0.0399), and P4 and P6 (P=0.0231). * denotes statistically significant difference. Data is presented as mean ± SEM.

5.3.3 Effect of nitric oxide in cell viability

No statistically significant effect on cell viability was observed between treated and non-treated ECFCs (P=0.6623) or between passages (P=0.2244). Similarly, no overall treatment effect was observed for CysNO treated ECs versus non-treated (P>0.9999) (Figure 17).

5.3.4 Effect of nitric oxide in cell growth

Cell growth parameters were not different between CysNO treated and untreated ECFCs at any passage. The NCD was not significantly different between treatment groups at any passage (P=0.7913), and no difference between passages was observed (P=0.0949). No difference in NCD between treated and untreated ECs was observed (P=0.8413). Similarly, no statistically significant difference was observed between ECFCs treated with CysNO or EGM alone for PDT at any timepoint (P=0.7895) or between passages (P=0.1113). No difference in PDT between treated and untreated ECs was observed (P>0.9999) (Figure 18). When evaluating the CPDL, significant difference between passages were observed for both treated and untreated ECFCs (P=0.0002). However, no difference was observed between treatment at any passage (P=0.9050). No difference in CPDL between treated or untreated ECs was observed (P=0.6905).

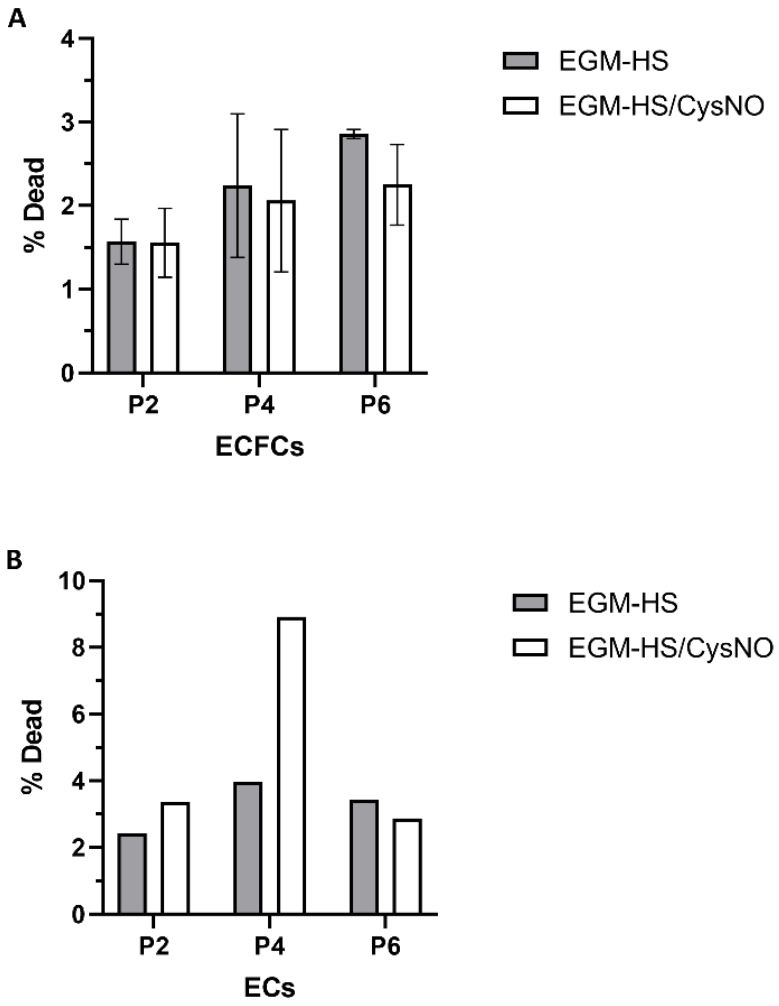


Figure 17. Effect of NO in cell viability for equine ECFCs and ECs. Percentage of dead cells at 3 different passages for A) ECFCs or B) ECs exposed to either 100 μM of CysNO (EGM-HS/CysNO), or EGM-HS alone. Data is presented as mean ± SEM.

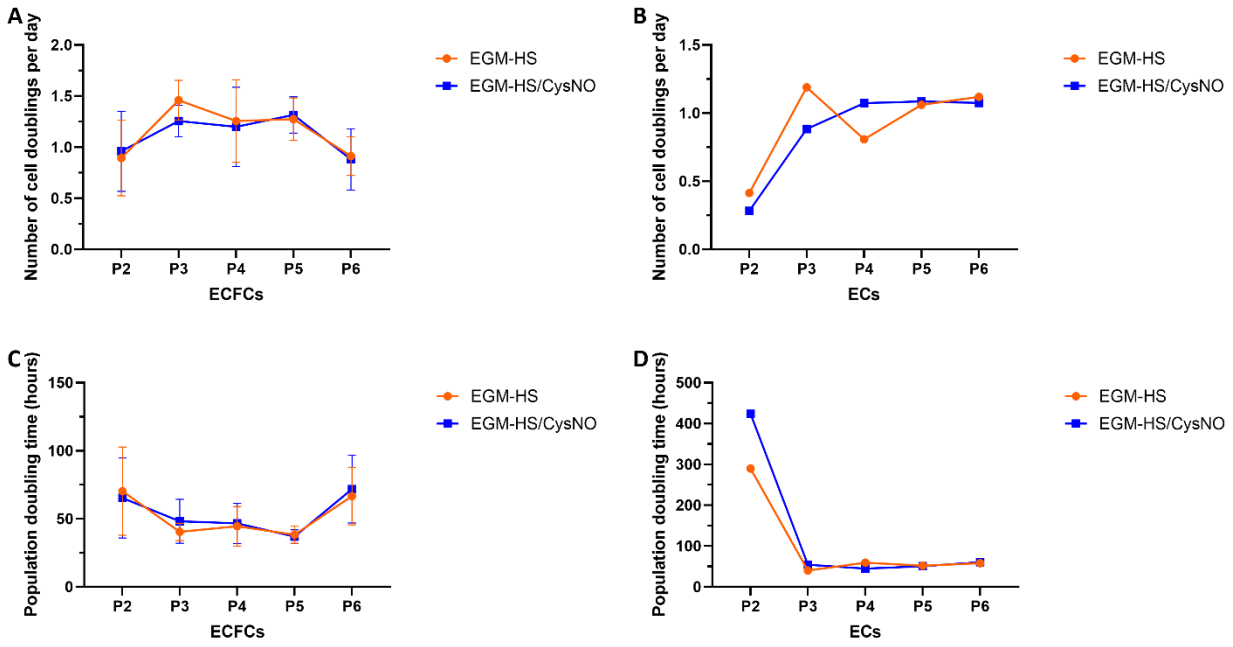
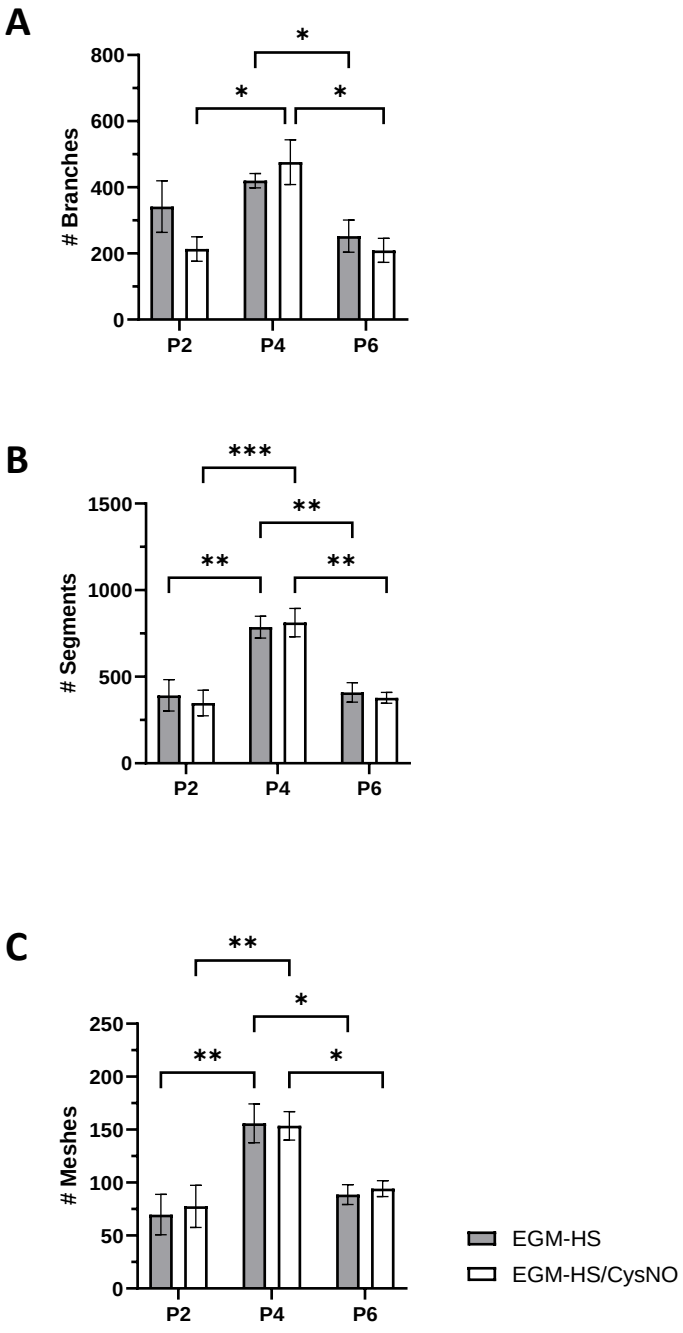


Figure 18. Effect of NO in cell growth kinetics. Number of cell doublings for A) ECFCs and B) ECs, and population doubling time for C) ECFCs and D) ECs between P2-P6. Data is presented as mean ± SEM.

5.3.5 Effect of nitric oxide in in vitro tubule formation

Passage number had a significant effect on all parameters of tubule formation for both the EGM-HS and EGM-HS/CysNO groups (P<0.0001). When comparing P4 with P2 within the EGM-HS and EGM-HS/ CysNO groups, greater number of: segments (P=0.0015, P=0.0010 respectively), branches (P=0.0169 only for EGM-HS/CysNO), meshes (P=0.0031, P=0.0071 respectively), junctions (P=0.0029, P=0.0013 respectively), tube length (P=0.0007, P=0.0007 respectively), and total mesh area (P=0.0005, P=0.0396 respectively) was observed in P4 when compared to P2 (Figure 19).

Similarly, when comparing P6 with P4 within the EGM-HS and EGM-HS/ CysNO groups, greater number of: segments (P=0.0018, P=0.0085 respectively), branches (P=0.0240, P=0.0446), meshes (P=0.0205, 0.0231), junctions (P=0.0016, P=0.0089 respectively), tube length (P=0.0168, P=0.0061 respectively), and total mesh area (P=0.0020, P=0.0044 respectively) was observed in P4 when compared to P6. However, no effect of treatment was observed at any timepoint between groups for number of segments (P=0.8605), number of branches (P=0.4233), meshes (P=0.7624), junctions (P=0.7148), tube length (P=0.1025), or total mesh area (P=0.9136) (Figure 19).


A
800
600
400
200
0
Branches
P2
P4
P6
*
*
*
B
1500
1000
500
0
Segments
P2
P4
P6
***
**
**
**
C
250
200
150
100
50
0
Meshes
P2
P4
P6
**
*
**
*
EGM-HS
EGM-HS/CysNO

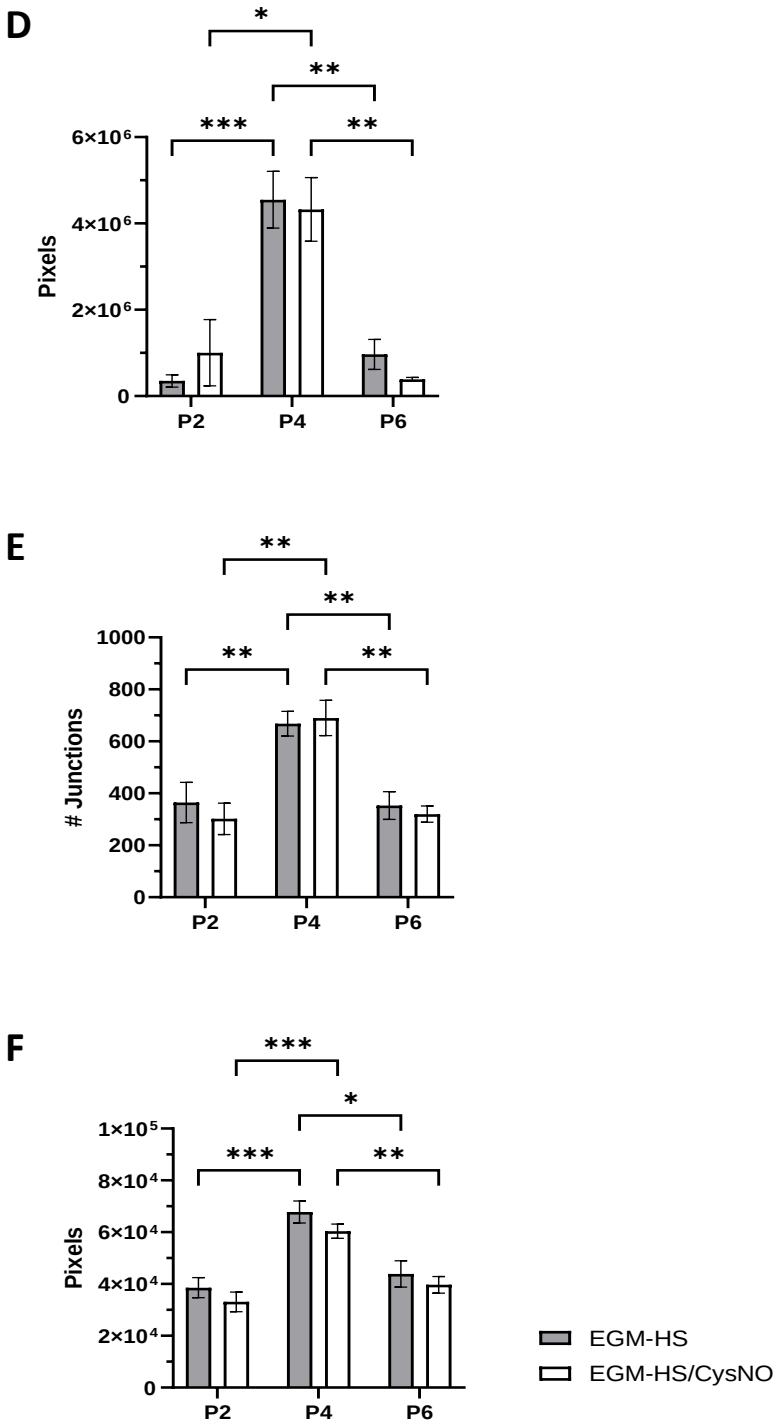
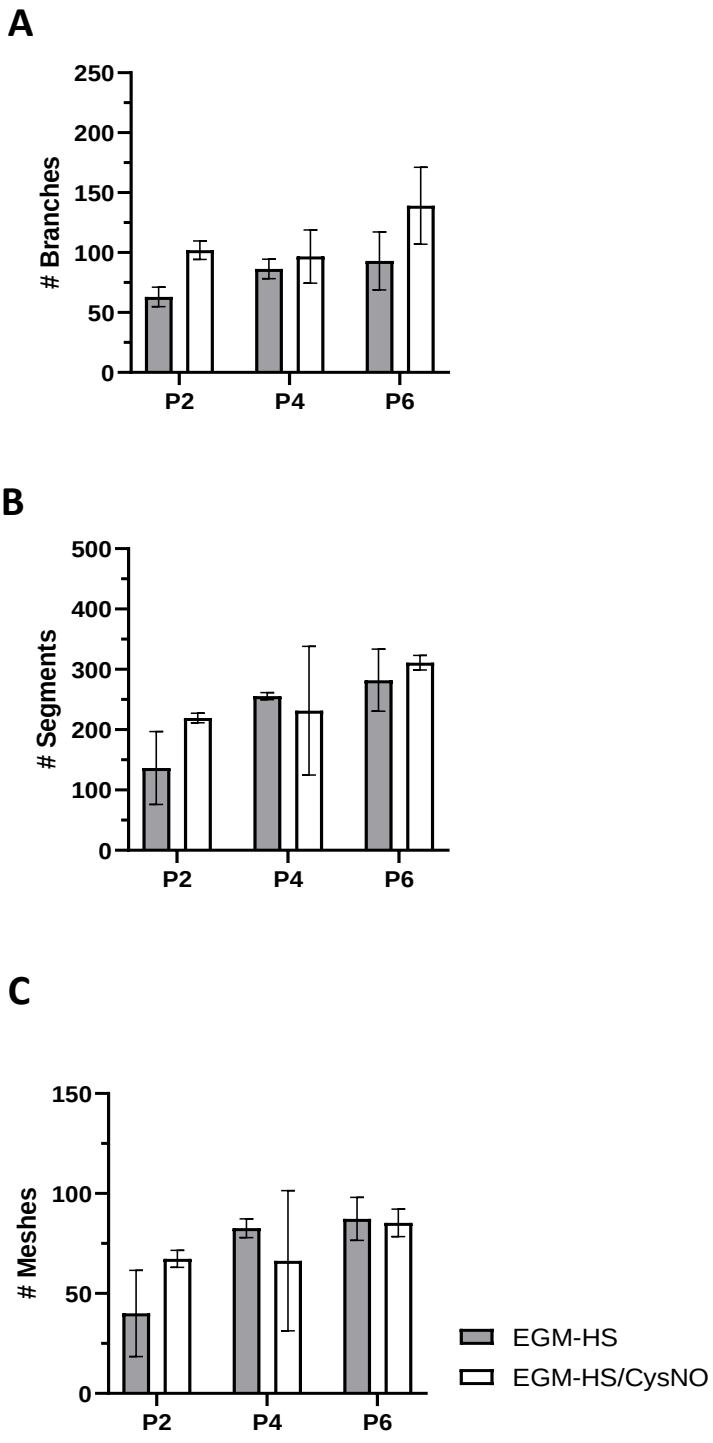


Figure 19. Effect of nitric oxide in *in vitro* tubule formation of ECFCs at different passages. A) number of branches, B) number of segments, C) number of meshes, D) tube length, E) number of junctions, and F) total mesh area. Statistically significant difference between passages for each group is represented by *. Data is presented as mean ± SEM.

When comparing ECs exposed to CysNO or EGM-HS alone, no significant effect of treatment in the number of segments (P=0.4561), number of branches (P=0.1908), number of meshes (P=0.7986), number of junctions (P=0.3254), tube length (P=0.3624), or total mesh area (P=0.5837) was observed at any passage (Figure 20).


A
250
200
150
100
50
0
Branches
P2
P4
P6
B
500
400
300
200
100
0
Segments
P2
P4
P6
C
150
100
50
0
Meshes
P2
P4
P6
EGM-HS
EGM-HS/CysNO

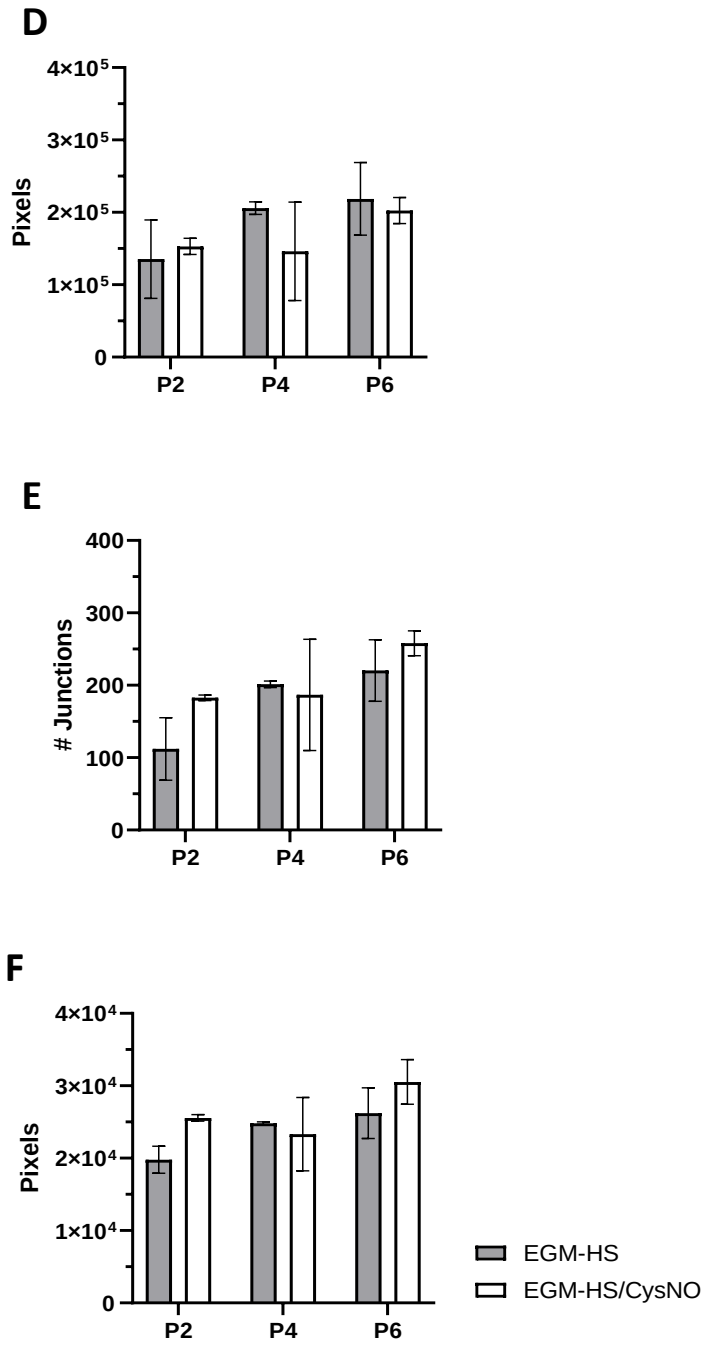


Figure 20. Effect of nitric oxide in *in vitro* tubule formation of ECs at different passages. A) number of branches, B) number of segments, C) number of meshes, D) tube length, E) number of junctions, and F) total mesh area. Data is presented as mean ± SEM.

5.3.6 Effect of nitric oxide in VEGF-A release

VEGF-A release in EGM-HS was below limits of detection (<0.06 ng/mL) for all samples. Concentrations of VEGF-A in EC lysate was 0.183 ng/mL and 0.112 ng/mL for groups containing 200 μM and 50 μM of CysNO respectively. VEGF-A concentration in the EC lysate for the group supplemented with 100 μM CysNO and L-NAME was 0.145 ng/mL. Data from the remaining EC lysate samples is not reported due to a high percentage (>10%) in the coefficient of variation.

5.4 Discussion

This study evaluated the effects of adding the NO donor CysNO to cultured equine ECFCs and its effect at different cell passages. Although an overall decrease in CD31 expression as the passage increased was observed for both the treated and untreated ECFCs, this decrease was statistically significant just for ECFCs supplemented with NO. A change in cell phenotype at different cell passages is not an uncommon finding, and similar to this study, human ECFCs have been observed to have a decrease expression in CD31 as the cell passage increases.[144, 171] This could be related to different factors, one being phenotypic shift driven by direct interactions, transfer of cell surface receptors or epigenetic reprogramming via transcriptional regulators from other cell types.[128, 129, 172, 173] Another factor could have been cell senescence, which at higher cell passages is known to cause deteriorations in cell integrity, function and karyotype, affecting the expression of cell surface proteins.[174] It is unclear why only the CysNO-treated ECFCs experienced a decrease in CD31 expression at P6 when compared to P2 and P4. It is unknown if equine ECFCs are more sensitive to the effects of a NO

donor when compared to ECs, making them more prompt to cell deterioration and alterations in their phenotype. In rat carotid arterial smooth muscle cells, it was observed that a specific cell phenotype was more sensitive than other to the cytotoxic effects of the NO donor SNAP.[175]

However, results in this study showed a very low expression of CD31 for equine ECFCs; therefore, the effects of NO on CD31 expression should be taken with caution. On the contrary, a high expression of CD31 was observed in equine ECs, which have been consistent with previous experiments. Although no significant difference was observed between treated and control ECs, or between passages, the sample size was small and only one cell line was included, thus precluding making any conclusion. Moreover, it is unknown if this percentage of expression will change with passages beyond P6, like it has been reported with mature primary human EC *in vitro*.[176]

Our preliminary viability analysis showed no significant difference in the percentage of dead ECs or ECFCs between the 3 different CysNO concentrations. However, an increase trend in percentage dead cells was observed with higher CysNO concentrations. An unexpected result from this viability analysis was the fact that the mean percentage of dead cells was significantly higher for ECFCs compared to ECs. Other factors like the growth media type, cell density at the time of dissociation or previous freezing techniques might have affected cell viability of ECFCs.[177, 178] In the present study, since cryopreserved cells were used, it is unknown if different techniques or concentrations of the permeating agent dimethyl sulfoxide were used between ECFCs and ECs. Even though the beneficial signaling effects of NO in cellular function are dose dependent, at higher concentrations it will elicit a cytotoxic effect and it is unknown if

equine ECFCs are more prompt to cytotoxic effect of NO compared to equine ECs. Nonetheless, the concentration of 100 μM used here has been used in other studies with no detrimental effect in cell viability, but an increase in cell growth and function.[92] Moreover, the increase in percentage of dead ECFCs was also observed in the untreated group, therefore this could have been related to other factors common to both study groups such as passage-induced senescence, rather than the addition of NO.[179] A concentration of 100 μM CysNO was elected based on these preliminary results and a report by Hodge *et al*.,[92] showing that no difference in cell viability was observed in cardiomyocytes treated with 100 μM or 50 μM CysNO. Furthermore, the use of 100 μM resulted in better spontaneous contraction.[92]

The addition of a NO donor to equine ECFCs in culture did not have a significant effect on growth kinetics. Moreover, the proliferative activity was not affected, which is an important prerequisite for cell expansion. These results were consistent with a previous study using equine ECFCs, where an initial decline in PDT was observed, followed by a slow increase from passage 5 to 8.[31] When analyzing the CPDL, results from the present study are in agreement with studies using human MSCs, where from P3 to P6, a persistent increase has been observed. The NCD observed in the present study was consistent as well with a previous report using equine ECFCs, where after an initial increase, a sustained decline in NCD was observed from passage 5 to 10.[28, 31]

Nitric oxide is known to positively mediate angiogenesis, thus, an increase in growth kinetics as well as greater tubule formation *in vitro* was expected in this study. However, the results obtained are in contrast with some studies using human ECs exposed to NO donors,

where a dose-dependent increase in number of branches was observed.[180] Similarly, the use of a sustained release method to deliver a NO donor to cultured human umbilical vein ECs resulted in significantly larger number of tubules when compared to the use of EGM-2 alone.[181] However, these studies evaluated the ECs function only at one timepoint, without studying the overall effect of passage number in tubule formation. Here, tubule formation at P4 was superior in all the parameters evaluated when compared to P2 and P6, which is in agreement with various reports evaluating *in vitro* tubule formation in human ECs and ECFCs, where cells from passages 2 to 6 were optimal for the assay.[171, 182] However, different studies have had different results regarding the effect of passage number in *in vitro* tubule formation, and a report concluded that human bone marrow derived EPCs had better *in vitro* tubule formation capacity at P12 when compared to P7.[183]

A limitation of our study was the fact that endogenous NO production was not blocked. This would have aid in determining if NO, either exogenous or endogenous, could have influence ECFC growth, phenotype or function *in vitro*. Furthermore, the release of NO was not measured beyond the first hour, where a high release of about 90% was observed. Thus, it is unknown if redosing before the 48 hours was necessary. Even though it was demonstrated NO release from the donor used, it is unknown if equine ECFCs or ECs were able to uptake the NO. Nitric oxide has been determined to enhance the synthesis of VEGF-A in human ECs, most likely through the non-hypoxic induction of HIF-1α, which is one of the most important factors to stimulate angiogenesis. Therefore, VEGF-A concentrations can be measured in growth media and cell lysate as a mean to assess the effect of supplementing NO in the cell culture media.[89] Preliminary results from the current study suggests no change in the VEGF-A concentrations in

cell lysates when adding a NO donor, however most of the concentrations obtained were below the limits of detection and precluded any conclusions.

Although the addition of NO to cultured equine ECFCs has no effect in the expression of the endothelial cell marker CD31, it was demonstrated that the use of 100 μM CysNO as a NO donor has no detrimental effect on cell viability, growth kinetics or function, which is of importance for future studies using NO in culture equine ECFCs. Further studies investigating alternative drug delivery methods such as polymers for sustained release of NO, like it has been described for human ECs could be more efficacious in maintaining steady concentrations of NO in cultured equine ECFCs.[184] Furthermore, research evaluating the long-term release profile of NO from CysNO in cultured equine ECFCs, its effects in VEGF release from treated cells, as well as *ex vivo* assays to evaluate the incorporation of NO-treated ECFCs into neovessels are warranted to better evaluate the potential use of NO in equine ECFCs.

Chapter 6 - Summary and conclusions

Phenotypic characterization of equine ECFCs continues to be a challenge, and changes in their phenotype while in expansion in culture is highly likely. The low expression of the EC marker CD31 together with high levels of the monocytic surface protein CD14 may be an indication that additional cell types are being isolated, thus over growing the ECFCs or triggering a phenotypic shift. The use of cell sorting techniques is possible in equine ECFCs when appropriate protocols are used to reduce cell clumping and increase efficiency. Cell sorting is able to isolate population of ECFCs based on the uptake of Ac-LDL and expression of CD31; however, since Ac-LDL and CD31 are not unique of ECFCs and may be shared with other cell groups, further expansion of sorted populations results in the loss of the desired phenotype. Refinement of sorting techniques for ECFCs, such as the use of a combination of different cell surface markers, or sorting combined with manual cell selection might overcome the challenges observed in this study; however, the lack of equine specific antibodies further complicates these techniques.

The modification of the mouse aortic ring assay, commonly used for *ex vivo* angiogenesis studies, can be used with equine arteries. This assay serves as a step further from *in vitro* angiogenesis function tests, by incorporating supporting vascular cells like seen *in vivo*, giving a close representation of the angiogenic process in live horses. However large variability, most likely inherent to the horse was observed. The preselection of rings after sprouting has started in serum-free media was shown to reduce part of this variability and is recommended for future use of this technique. This arterial ring assay was very versatile and responded differently to different sources of growth factors and has the potential to be used in future

studies assessing the function of equine ECFCs in a co-culture assay. Although angiogenesis using ePL was not superior to the traditional HS, it proved to be an alternative source of equine-specific growth factors to support angiogenesis. Moreover, other biologics such as PPP may also be used in cell culture media for the support of cell growth, however more research is needed to determine the benefits of both PPP and ePL in the culture of equine ECFCs. The analysis of results from this angiogenesis assay can be done by image modification using computer software, which provides a more objective and less time-consuming way to quantify the different parameters of vessel growth.

To overcome some of the downsides of static cell culture, like the absence of shear forces, the addition of a NO donor to the EGM was studied. The results of this study did not show any improvement in the phenotype or angiogenic function of equine ECFCs after the addition of NO to the culture media. Moreover, these cells might be more sensitive to the effects of NO at the dose used, since a higher percentage of dead cells was recorded when compared to equine carotid ECs. The use of CysNO at a lower concentration or by a more sustained release method may be needed. Nonetheless, the NO donor CysNO does not cause a significant detrimental effect in cultured equine ECFCs, and growth kinetics parameters and *in vitro* angiogenesis was maintained. Based on the positive effects of NO in the culture of other cell types, using NO donors together with other ways to improve cell culture like the use of alternative sources of growth factors and cell sorting techniques may improve cell culture outcomes. Moreover, the resultant angiogenic effects following modifications to the isolation and culture of equine ECFCs could be effectively evaluated by the use of equine arteries ex vivo, thus bringing new options in the study of angiogenesis in horses.

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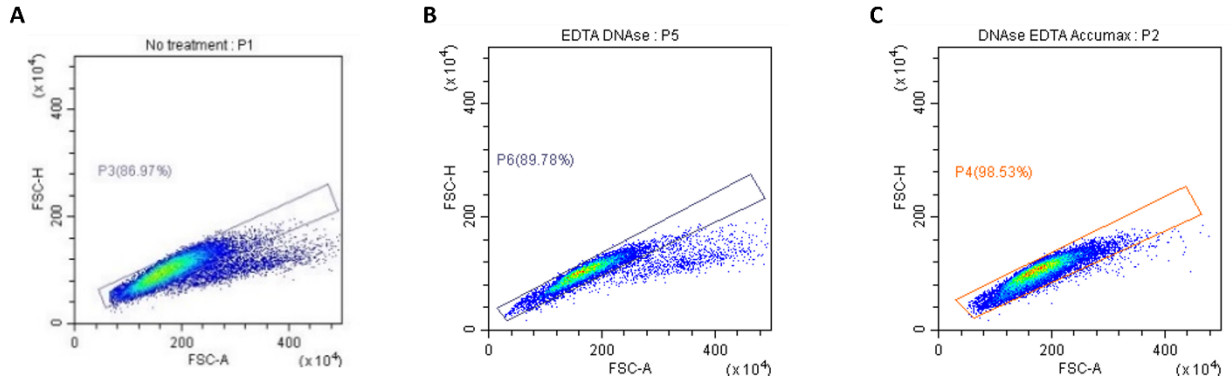
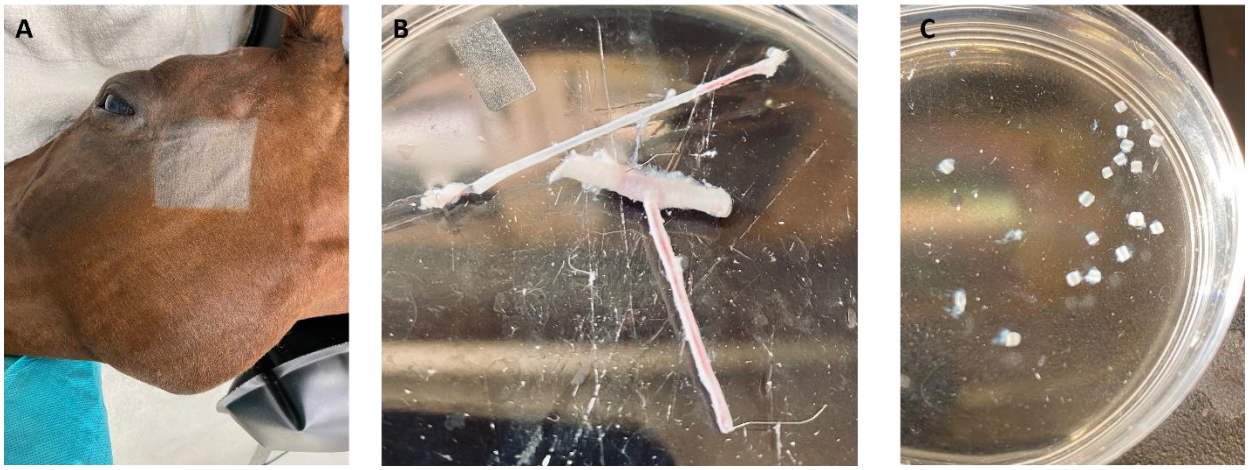
Appendix A – Preliminary research for determination of cell clumping following different protocols for FC



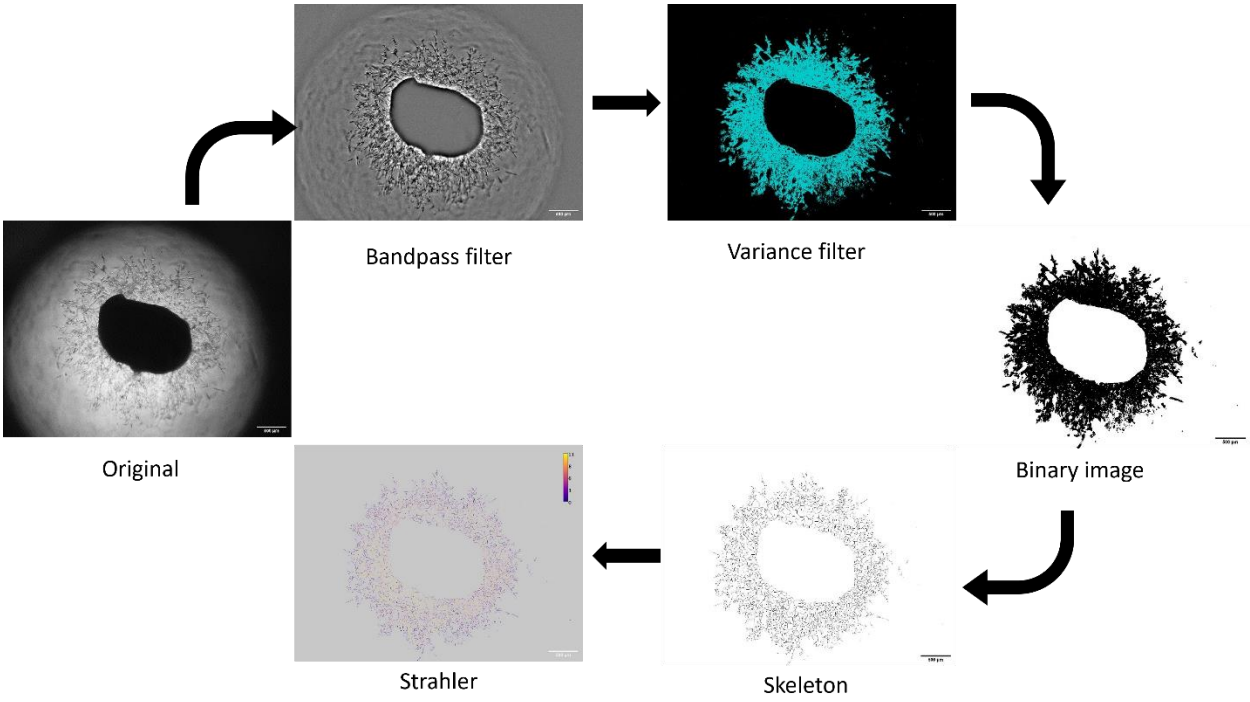
Results from FC analysis of cells under different treatments to reduce cell clumping. A) ECFCs with no treatment added and resuspended in 1% BSA alone. B) Cells resuspended in 1% BSA containing EDTA and DNAse. C) ECFCs resuspended in 1% BSA with EDTA, DNAse and Accumax™ added. Gating includes percentage of singlets, excluding cell clumps.

Appendix B – Protocol for facial arterial harvesting and dissection



Arterial ring preparation. A) Skin at the facial artery site was aseptically prepared, and a section containing the facial artery and branches was excised and further dissected. B) Arterial dissection of branches of the facial artery measuring 1 mm in diameter. C) Arterial rings of 1 mm length ready to be embedded in Matrigel®.

Appendix C – Sequence of image modification for isolation of a vascular tree

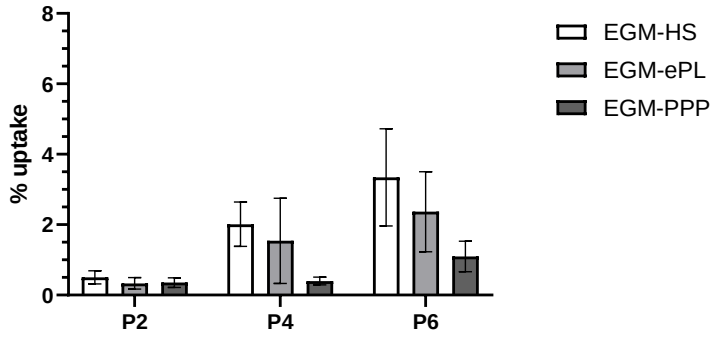


Sequence of steps in the image modification process using Fiji software to isolate the vascular tree in the arterial ring assay. From the original photomicrograph, 2 different filters were used to create a binary image of the vascular tree. Finally, a Strahler analysis was used to quantify the number of branches.

Appendix D – Pilot study to evaluate the effects of equine platelet lysate in cultured ECFCs

This pilot study was done with the purpose of evaluating the effects of ePL on ECFCs expansion to consider its use in the evaluation of the NO donor in cultured ECFCs.

Cryopreserved ECFCs from 3 different horses were cultured in EGM containing 10% of either: 1) HS (EGM-HS), 2) ePL (EGM-ePL), or 3) PPP (EGM-PPP). ECFCs were maintained from P2 to P6 and analyzed for the uptake of CD31 by FC and tubule formation in Matrigel®. The percentage uptake of CD31 was compared by 2-way repeated measures ANOVA with Tukey's post hoc test. Significance was set at $P \leq 0.05$.



Effect of HS, ePL or PPP on CD31 expression of cultured equine ECFCs at 3 different passages. Data is presented as mean ± SEM.

No statistically significant difference was observed between groups at any timepoint (P=0.37820). However, time had an overall significant effect on the CD31 uptake (P=0.0091).

Matrigel tubule formation in ECFCs for each of the study groups. A) EGM-ePL, B) EGM-HS, and C) EGM-PPP.

Cells from every group were able to form tubules in Matrigel® that appeared to be of similar magnitude. Based on this preliminary data, it was decided to study the effects of NO donor in ECFCs cultured with HS as the source of growth factors since it has been proven to be effective in the culture of these cells.