Veterinary Parasitology 318 (2023) 109939

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Involvement of the excretory/secretory and surface-associated antigens of *Dirofilaria immitis* adult worms in the angiogenic response in an *in-vitro* endothelial cell model

Cristian David Cardona Machado [a], Claudia Alarcón-Torrecillas [b], Miguel Pericacho [b], Iván Rodríguez-Escolar [a], Elena Carretón [c], José Alberto Montoya-Alonso [c], Rodrigo Morchón [a,*]

[a] *Zoonotic Diseases and One Health group, IBSAL-CIETUS (Biomedical Research Institute of Salamanca-Research Centre for Tropical Diseases University of Salamanca), Faculty of Pharmacy, University of Salamanca, 37007, Salamanca, Spain*

[b] *Department of Physiology and Pharmacology, Biomedical Research Institute of Salamanca (IBSAL), University of Salamanca, Salamanca, Spain*

[c] *Faculty of Veterinary Medicine, Research Institute of Biomedical and Health Sciences (IUIBS), University of Las Palmas de Gran Canaria, Arucas, 35413, Las Palmas, Spain*

ARTICLE INFO

Keywords:
Angiogenesis
Excretory/secretory antigens
Surface-associated antigens
Adult *Dirofilaria immitis* worms
Cell proliferation and migration
Pseudo-capillary formation

ABSTRACT

Angiogenesis is a process by which new vessels are formed from pre-existing ones when the physiological conditions of the vascular endothelium are altered. Heartworm disease, caused by *Dirofilaria immitis,* causes changes in the vascular endothelium of the pulmonary arteries due to obstruction, friction, and hypoxia. The aim of this study was to analyze whether the excretory/secretory and surface-associated antigens of adult worms interact and modulates the angiogenic mechanism, viable cell number and cell migration, as well as the formation of pseudo-capillaries. Cultures of human vascular endothelial cells (HUVECs) stimulated with excretory/secretory antigens (DiES), surface-associated antigens (Cut) from *D. immitis* adult worms, VEFG-A (Vascular Endothelial Growth Factor A), as well as DiES+VEFG-A and Cut+VEFG-A were used. The production of VEFG-A and other proangiogenic [soluble VEFGR-2 (sVEFGR-2), membrane Endoglin (mEndoglin)] and antiangiogenic [VEFGR-1/soluble Flt (sFlt), soluble Endoglin (sEndoglin)] molecules was assessed using commercial ELISA kits. Cell viability was analyzed by live cell count and cytotoxicity assays by a commercial kit. In addition, viable cell number by MTT-based assay, cell migration by wound-healing assay carrying out scratched wounds, and the capacity of pseudo-capillary formation to analyze cell connections and cell groups in Matrigel cell cultures, were evaluated. In all cases, non-stimulated cultures were used as controls. DiES+VEFG-A and Cut+VEFG-A significantly increased the production of VEFG-A and sVEFGR-2, and only Cut+VEFG-A significantly increased the production of VEFGR-1/sFlt compared to other groups and non-stimulated cultures. Moreover, only DiES+VEFG-A produced a significant increase in viable cell number and significant decrease cell migration, as well as in the organization and number of cell connections. Excretory/secretory and surface-associated antigens of adult *D. immitis* activated the angiogenic mechanism by mainly stimulating the synthesis of proangiogenic factors, and only excretory/secretory antigens increased viable cell number, activated cell migration and the formation of pseudo-capillaries. These processes could lead to vascular endothelial remodeling of the infected host and favor the long-term survival of the parasite.

1. Introduction

Angiogenesis is the formation of new blood vessels from pre-existing vessels, a key process in the resolution of physiological situations such as wound healing and obstructive inflammatory processes, among others. Angiogenesis begins with tissue hypoxia/ischemia, which leads to increased growth, proliferation and differentiation of vascular endothelium. These alterations produce physiological effects such as cardiac ischemia, hypertension, or respiratory distress (Hershey et al., 2001, Van et al., 2001, Eilken, Adams, 2010). These physiological alterations upregulate the expression of different genes involved in various steps of angiogenesis mediated by the balance and interaction between

* Corresponding author.
E-mail address: rmorgar@usal.es (R. Morchón).

https://doi.org/10.1016/j.vetpar.2023.109939
Received 21 March 2023; Received in revised form 23 April 2023; Accepted 25 April 2023
Available online 27 April 2023

numerous pro- and anti-angiogenic factors (Tahergorabi et al., 2012). Vascular Endothelial Growth Factor (VEFG-A), at which point migration, endothelial cell proliferation and tube formation begin (Phng, Gerhardt, 2009, Gerhardt et al., 2003), is the most potent angiogenic factor and is the first produced in "endothelial tip cells", which enables them to begin proliferation and migration and orient themselves according to the VEFG-A gradient produced, mediated by the interaction of VEFG-A-Receptor 2 (VEFGR-2) and soluble VEFGR-2 (sVEFGR-2), at which point migration, endothelial cell proliferation and tube formation begin (Gerhardt et al., 2003). This process ends with the inhibition of endothelial proliferation and migration and formation of new capillaries mediated by platelet-derived growth factor β (PDGF-B) and its receptor (PDGFR-B) (Betsholtz, 2004). However, both VEFGR-1 and its soluble form (sFlt1) exert negative regulation of signalling through VEFGR-2, as they act by sequestering the ligand and preventing it from binding to the receptor (Hazarika et al., 2007).

The other main molecule is endoglin, a transmembrane glycoprotein, essential for angiogenesis, cell proliferation and vascular development, which is predominantly expressed in vascular endothelial cells and the suppression of which produces the inhibition of angiogenesis (Liu et al., 2014). Several endoglin isoforms have been described. One of them, the circulating form of endoglin, soluble endoglin (s-Endoglin), negatively regulates the production of several proangiogenic and promigratory proteins involved in angiogenesis (Gregory et al., 2014, Gallardo-Vara et al., 2018, Varejckova et al., 2017). However, the membrane-bound form (m-Endoglin) is involved in processes with proangiogenic effects (Ollauri-Ibáñez et al., 2017).

Heartworm disease is a vector-borne zoonotic disease, caused by *Dirofilaria immitis*, mainly affecting domestic and wild canines and felines, with the main reservoir being the domestic dog (Morchón et al., 2022). Adult worms are found in the pulmonary artery and right ventricle of the heart where they can live for several years. Their presence causes chronic pathology due to anatomical and histological changes in the arterial walls causing proliferative endarteritis, characterized by a loss of elasticity, decreased arterial lumen and increased permeability of the vascular wall. These changes can lead to hypoxia, pulmonary hypertension and congestive heart failure. When the adult worms die, an acute pathology occurs as a result of the massive release of antigens from the parasite and the endosymbiont bacteria of the genus *Wolbachia*, which may increase clot formation (pulmonary thromboembolism), exacerbate endothelial and lung damage, accelerate heart failure, or cause the death of the infected host (Simón et al., 2012, Ames & Atkins, 2020). Moreover, microfilariae, which can live up to 2 years (Simón et al., 2012), are found in the glomeruli and can cause renal dysfunction (Morchón et al., 2012).

Dirofilaria immitis participates in several processes that contribute to its survival within the host, limiting short-term damage, although long-term vascular damage is inevitable (Dillon et al., 2008). Excretory/secretory antigens promote vasodilation (Morchón et al., 2010) and in the short term regulate the host fibrinolysis system by preventing or promoting the lysis of fibrin clots (González-Miguel et al., 2012, Diosdado et al., 2020), as well as cuticle surface-associated antigens of adult worms (González-Miguel et al., 2013). In the long term, plasmin production increases *in-vitro* endothelial and muscle proliferation, as well as migration and destruction of extracytoplasmic matrix, all of which are related to vascular remodeling (González-Miguel et al., 2015). On the other hand, when adult worms die, naturally or induced by adulticidal treatment, *Wolbachia* somatic antigens promote a vasoconstrictive and chemotactic endothelial response that activates anti-angiogenic mechanisms and inhibits *in-vitro* formation of pseudo-capillaries; this seems to be related to the amount of *Wolbachia* antigens released into the bloodstream (Morchón et al., 2008, Zueva et al., 2020).

Taking into account that *D. immitis* is involved in the modulation of inflammatory mechanisms that allow the parasite to survive in the host for years, the aim of this study was to analyze the influence of excretory/secretory and surface-associated antigens of adult *D. immitis* in the production of some factors involved in the angiogenic process, in the viable cell number and migration, and in the formation of pseudo-capillaries, using an *in-vitro* model of vascular endothelial cells.

2. Methods

2.1. Reagents

Excretory/secretory antigens of adult *D. immitis* (DiES) were prepared as previously described (González-Miguel et al., 2012). In brief, 23 live worms (8 males and 15 females) obtained from a naturally infected dog from Canary Islands were washed in sterile PBS pH 7.2 and incubated for 1 day in 50 ml of Eagle's minimum essential medium (MMEE) (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 0.04 % gentamycin and 0.01 % nistatin, at 37 ºC. Later, it was dialyzed against 0.01 % PBS pH 7.2 and filtered through an Amicon YC05 (Amicon Corporation Scientific System Division, Danvers, MA, USA). The final concentration obtained was 0.4 μg/μl.

Cuticle antigens of adult *D. immitis* (Cut) were obtained following the methodology described by Wedrychowicz et al. (1994). In brief, 10 live worms (4 males and 6 females) obtained from a naturally infected dog from Canary Islands were washed and then incubated in saline solution containing 0.25 % cetyltrimethylammonium bromide (CTAB) with a cocktail of protease inhibitors (Morchón et al., 2014) at 37 ºC for 4 h. The worms were separated from detergent and extracted proteins were precipitated with sodium acetate 0.002 mM with nine volumes of 96 % ethanol, at −20 ºC for 48 h followed. Finally, Cut was centrifuged at 10, 000xg during 10 min and the resulting pellets were resuspended in PBS pH 7.2.

Protein concentration of both antigens (DiES and Cut) were measured by detergent compatible (DC) protein assay commercial kit (Bio-Ras) and were stored at −80 ºC until use.

2.2. Cell culture and stimulation of endothelial cells

Human Umbilical Vein Endothelial Cells (HUVECs) were grown in Endothelial Basal Medium 2 (Lonza, Walkersville, MD, USA) supplemented with SingleQuots® (Lonza, Walkersville, MD, USA): 20 % fetal bovine serum, heparin (22.5 μg /ml), VEFG-A (0.5 ng/ml), ascorbic acid (1 μg /ml), hFGF-B (10 ng/ml), hydrocortisone (0.2 μg /ml), hEGF (5 ng/ml), Gentamicin (30 mg/ml), amphotericin B (15 μg/ml) and R^3-IGF-3 (20 ng/ml). Plates were pre-coated with 0.1 % pig gelatine (Sigma Chemical Co., San Luis, USA), 0.01 % fibronectin (SigmAldrich, Misuri, USA) and 0.01 % collagen (Corning). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5 % CO_2/95 % air. The medium was changed every 3 days. Expansion was carried out by trypsinizing the cells (Trypsin/EDTA, Lonza, USA) and replating them when the proliferating cells had reached sufficient density. Passaging was performed the ratio of 1:3. Cell counts were performed using a Countess® Automated Cell Counter (Invitrogen, California, USA) following the manufacturer's instructions.

HUVECs were treated as previously described (Morchón et al., 2008, Pérez Rodríguez et al., 2023). In brief, endothelial cells (10^6 cells/plate) were plated on 60 mm culture plates and were grown for 4 days to obtain confluent cultures and treated with 5 different stimuli: 1 μg/ml of DiES, Cut or recombinant Vascular Endothelial Growth Factor protein (VEFG-A) (RRD SYSTEMS), 1 μg/ml of DiES plus 1 μg/ml of VEFG-A and 1 μg/ml of Cut plus 1 μg/ml of VEFG-A. Non-stimulated cells were used as controls in the same conditions. Finally, the supernatant of the cell cultures was collected and HUVECs were lysed in ice-cold lysis buffer [20 mM Tris-HCl (pH 7.5); 140 mM NaCl; 10 mM ethylenediaminetetraacetic acid; 10 % glycerol; 1 % Igepal CA-630; aprotinin, pepstatin, and leupeptin at 1 μg/ml each; 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium orthovanadate].

2.3. Cell viability and cytotoxicity assays

Cell viability was analyzed through cell counts using the equipment Countess® Automated Cell Counter (Invitrogen) following the manufacturer's instructions. Cytotoxicity was assessed in the supernatant of the stimulated and control cell cultures by the Toxilight BioAssay Kit (Cambrex, Verviers, Belgium) following commercial instructions. This commercial kit quantitatively measures the release of adenylate kinase from damaged cells. The results were presented as the mean ± standard deviation (SD) of three experiments performed in duplicates.

2.4. Angiogenic factors assays

VEFG-A, soluble VEFGR-1 (VEFGR-1/sFlt), soluble VEFGR-2 (sVEFGR-2) and sEndoglin (sEng) concentration in the HUVECs culture medium were measured by ELISA using a Human VEFG-A Quantikine ELISA kit (R&D Systems, Minneapolis, USA), Human VEFGR-1/sFlt Quantikine ELISA kit (R&D Systems, Minneapolis, USA), Human sVEFGR-2 Quantikine ELISA kit (R&D Systems, Minneapolis, USA) and Human Endoglin Quantikine ELISA kit (R&D Systems, Minneapolis, USA) respectively; mEndoglin (mEng) concentration in the listed endothelial cells was measured by Human Endoglin Quantikine ELISA kit (R&D Systems, Minneapolis, USA) following the manufacturers' instructions for the first 24 h. The results were presented as the mean ± SD of three experiments performed in duplicate.

2.5. Viable cell number assays

Viable cell number assays were assessed as previously described (Zueva et al. 2020), with some modifications. In brief, 1000 cells per well were seeded on a 96-well plate in HUVECs culture medium with 5 different stimuli for 10 days: 1 μg/ml of DiES, Cut or Vascular Endothelial Growth Factor (VEFG-A) (RRD SYSTEMS), 1 μg/ml of DiES plus 1 μg/ml of VEFG-A and 1 μg/ml of Cut plus 1 μg/ml of VEFG-A. Non-stimulated cells were used as controls in the same conditions. Viable cell number was estimated every 2 days up to a maximum of 10 days of culture was estimated by incubating cell cultures with 0.5 mg/ml 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide (MTT) (SigmAldrich, St. Louis, MO, USA) for 4 h. Then, 10 % SDS in 0.01 M HCl was added at a 1:1 (v/v) ratio and left overnight at 37 °C. Finally, absorbance was measured at 570 nm. The results are presented as the mean ± SD of three experiments performed in triplicate.

2.6. Migration assays

Wound-healing assays were assessed as previously described by Pérez Rodríguez et al. (2023) with some modifications. In brief, *in-vitro* scratched wounds were created by scraping confluent cell monolayers in 60 mm sterile plates with a sterile disposable pipet tips. The remaining cells were washed with sterile PBS buffer and incubated with HUVECs culture medium with 5 different stimuli up to 6 h. Non-stimulated cells were used as controls in the same conditions. Endothelial cell migration into the denuded area was monitored by photography of the plates every 30 min. The results were presented as the mean ± SD of three experiments performed in triplicate.

2.7. Endothelial cell tube formation assay

Endothelial cell tube formation was assessed as previously described by Jerckic et al., (2006), with modifications. In brief, a total of 8000 HUVECs per well were plated on Matrigel® precoated μ-Slide Angiogenesis® plates (Ibidi, Gräfelfing, Germany) in HUVECs culture medium with 5 different stimuli: DiSA, Cut, VEFG-A, DiES+VEFG-A and Cut+VEFG-A (1:10 dilution). After seeding on Matrigel®, cells spread and aligned with each other to develop hollow, tube-like structures. The cells and intercellular junctions were observed each 30 min for 3 h of incubation and the morphological changes were photographed using a phase contrast inverted Leica Microscope (Leica, Wetzlar, Germany). Subsequently, the intercellular junctions were divided between the cell bodies to calculate the relationship between them (endothelial cell tube formation = cellular connections/cellular bodies). Non-stimulated cells were used as controls in the same conditions. Each experiment was performed in triplicate.

2.8. Statistical analysis

The GraphPad Prism v.7 was used for all data analyses. Analyses were performed by ANOVA and corrected for repeated measurements when appropriate. If ANOVA revealed overall significant differences, individual means were evaluated post-hoc using Tukey's test. All results were expressed as the mean ± SD. In all experiments, a significant difference was defined as a P-value of < 0.01 for a confidence level of 99 %.

3. Results

3.1. Effect of DiES and Cut antigens on cell viability and cytotoxicity

No differences were found in cell viability (Fig. 1) and cytotoxicity (date not shown) of stimulated cultures with DiES, Cut, VEFG-A, DiES+VEFG-A or Cut +VEFG-A compared to non-stimulated cell cultures.

3.2. Effect of DiES and Cut antigens on angiogenic factors

3.2.1. VEFG-A

The stimulation of cell cultures with DiES+VEFG-A significantly increased the production of VEFG-A when compared to cell cultures stimulated with DiES ($t = 175.5$, $df=4$, $P < 0.0001$) and VEFG-A ($t = 66.87$, $df=4$, $P < 0.0001$) and non-stimulated cultures ($t = 139.8$, $df=4$, $P < 0.0001$) for the first 24 h. In addition, VEFG-A-stimulated cell cultures showed a significant increase in VEFG-A production compared to DiES ($t = 22.31$, $df=4$, $P < 0.0001$) and non-stimulated cultures ($t = 18.97$, $df=4$, $P < 0.0001$). The stimulation of cell cultures with Cut+VEFG-A significantly increased the production of VEFG-A when compared to cell cultures stimulated with Cut ($t = 10.86$, $df=4$, $P = 0.0004$), VEFG-A ($t = 4.68$, $df=4$, $P = 0.0094$) and non-stimulated cultures ($t = 13.94$, $df=4$, $P = 0.0002$). In addition, VEFG-A-stimulated cell cultures showed a significant increase in VEFG-A production compared to Cut ($t = 9.372$, $df=4$, $P = 0.0007$) (Fig. 2A). No significant differences between cell cultures stimulated with VEFG-A and culture medium with VEFG-A prior to cell stimulation were detected for the first 24 h, being obtained similar values.

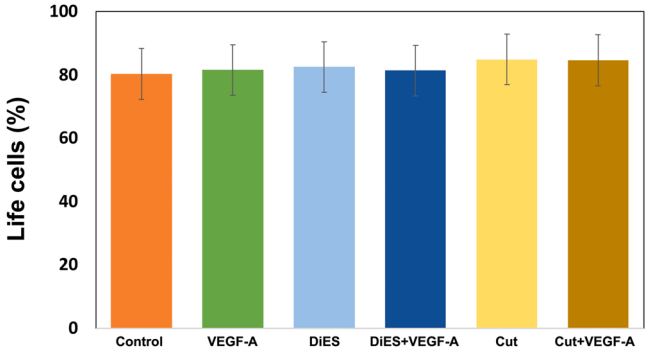

Fig. 1. Effects of DiES and Cut antigens on cell viability [percentage (%) of life cells] in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●) for 24 h. Results are expressed as the mean ± SD of 3 independent experiments.

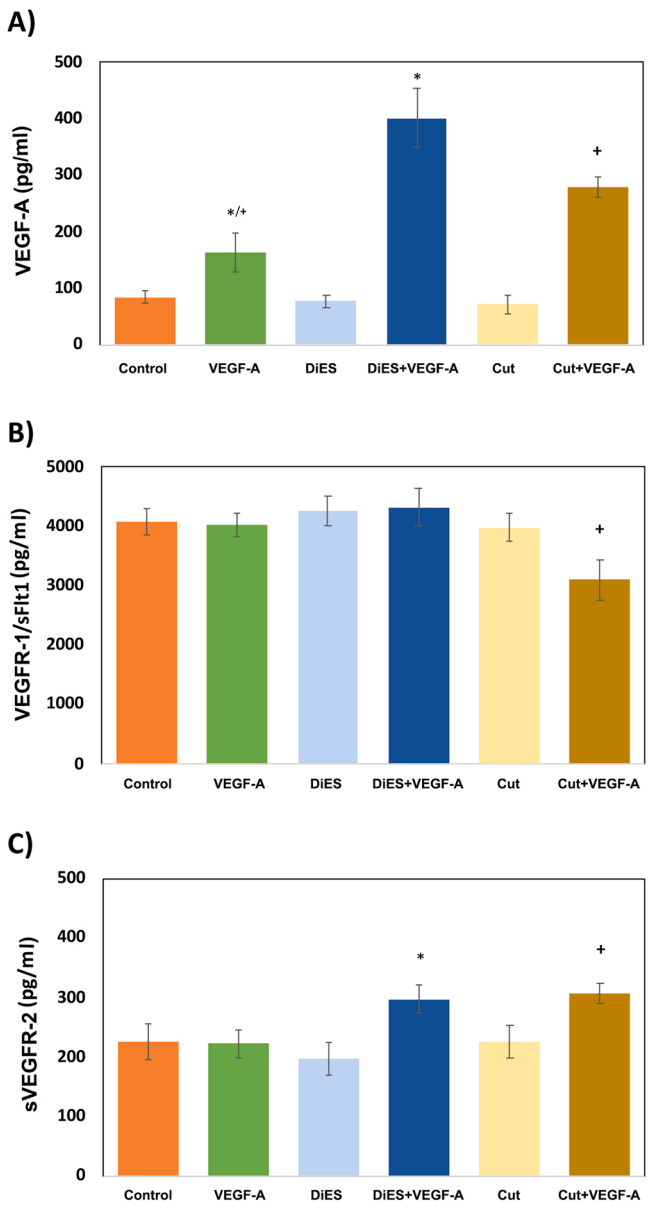

Fig. 2. Effects of DiES and Cut antigens on VEFG-A (A), VEFGR-1/sFlt1 (B) and sVEFGR-2 (C) in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●) for 24 h. Results are expressed as the mean ± SD of 3 independent experiments. The asterisk or plus (*/+) indicates significant differences ($p < 0.05$): DiES+VEFG-A *vs* control, VEFG-A and DiES (*) and Cut+VEFG-A *vs* control, VEFG-A and Cut (+).

3.2.2. *VEFGR-1/sFlt and sVEFGR-2*

On the one hand, with regard to the VEFGR-1/sFlt content detected, no significant differences were observed between DiES+VEFG-A, DiES, VEFG-A stimulated and unstimulated cell cultures for the first 24 h. However, a significant decrease was observed between Cut+VEFG-A stimulated cell cultures and Cut ($t = 4.390$, df=4, $P = 0.0118$), VEFG-A ($t = 14.88$, df=4, $P = 0.0001$) stimulated and unstimulated cell cultures ($t = 14.75$, df=4, $P = 0.0001$) (Fig. 2B). On the other hand, with regard to the sVEFGR-2 content detected, DiES+VEFG-A stimulated cell cultures showed a significant increase compared with DiES ($t = 5.826$, df=4, $P = 0.0043$), VEFG-A ($t = 6.151$, df=4, $P = 0.0035$) and non-stimulated cultures respectively ($t = 3.274$, df=4, $P = 0.0307$). In addition, Cut+VEFG-A stimulated cell cultures showed a significant increase compared with Cut ($t = 7.543$, df=4, $P = 0.0017$), VEFG-A ($t = 8.727$, df=4, $P = 0.0009$) and non-stimulated cultures respectively ($t = 4.047$, df=4, $P = 0.0155$) (Fig. 2C).

3.2.3. *mEndoglin and sEndoglin*

Regarding the sEndoglin production detected, no significant differences were observed between stimulated and unstimulated cultures for the first 24 h (Fig. 3). However, DiES+VEFG-A significantly increased the production of mEndoglin when compared to cell cultures stimulated with DiES ($t = 3.434$, df=4, $P = 0.0264$), VEFG-A ($t = 3.347$, df=4, $P = 0.0286$) and non-stimulated cultures ($t = 4.561$, df=4, $P = 0.0103$). A similar result was obtained when mEndoglin production was compared between cell cultures stimulated with Cut+VEFG-A and Cut ($t = 3.355$, df=4, $P = 0.0284$), VEFG-A ($t = 2.866$, df=4, $P = 0.0456$) and non-stimulated cell cultures ($t = 3.953$, df=4, $P = 0.0168$).

3.2.4. *DiES but not Cut antigens produces an increase in viable cells*

The effect of DiES or Cut and VEFG-A on the number of viable endothelial cells was analysed, estimating the viable cell number by MTT technique in a period of 10 days every 2 days (Fig. 4). All stimulated and unstimulated cell cultures showed typical curves of cell growth in all experimental groups with progressive growth between days 0 and 6 or 8 post-stimulation, experiencing a decrease in viable cells from there until day 10 post-stimulation. MTT technique showed a significant increase in the number of viable cells on day 6 post-stimulation in cultures stimulated with DiES+VEFG-A compared with cultures stimulated with DiES ($t = 9.072$, df=4, $P = 0.0008$), VEFG-A ($t = 3.778$, df=4, $P = 0.0195$) and unstimulated cultures ($t = 4.338$, df=4, $P = 0.0123$), and on day 8 post-stimulation in cultures stimulated with DiES+VEFG-A compared with cultures stimulated with DiES ($t = 15.27$, df=4, $P = 0.0001$), VEFG-A ($t = 4.764$, df=4, $P = 0.0089$) and unstimulated cultures ($t = 12.04$, df=4, $P = 0.0003$). However, there were no significant differences between cells stimulated with Cut+VEFG-A and the other stimulated groups (Cut and VEFG-A stimulated and unstimulated cultures).

3.2.5. *DiES but not Cut antigens produces cell migration*

A Wound Healing assay was performed to assess cell migration (Fig. 5). The quantification was carried out by measuring the distance of cell migration in comparison with negative control (untreated cells) to 6 h post-stimulation. A significant decrease of cell migration distance after stimulation with DiES+VEFG-A respect to DiES ($t = 18.83$, df=8, $P < 0.0001$), VEFG-A ($t = 9.249$, df=8, $P < 0.0001$) and unstimulated cultures ($t = 24.34$, df=8, $P < 0.0001$) was detected. However, cell migration distance in Cut, VEFG-A, Cut+VEFG-A stimulated and unstimulated cells was similar.

3.2.6. *Effect of DiES and Cut antigens on pseudo-capillary formation*

The capacity for pseudo-capillary formation was evaluated by analyzing the cell junctions (connections) and the cellular set (converging strengths of different cellular ramifications) that emerged in stimulated and unstimulated cell cultures (Fig. 6). The formation of pseudo-capillaries and the connections/set relationship in cultures stimulated with DiES+VEFG-A showed a significant increase for the first 3 h compared to cell cultures stimulated with DiES ($t = 7.974$, df=4, $P = 0.0013$), VEFG-A ($t = 9.127$, df=4, $P = 0.0008$) and non-stimulated cultures ($t = 10.67$, df=4, $P = 0.0004$). However, in the case of cultures stimulated with Cut+VEFG-A showed a moderate increase compared to cell cultures stimulated with Cut and VEFG-A and unstimulated cultures.

4. Discussion

Angiogenesis is an endothelial process that is triggered after stimulation by obstructive processes, resulting in increased vascular endothelial growth, cell proliferation and cell differentiation (Hershey et al., 2001; Eilken et al., 2019). The presence of adult *D. immitis* worms causes changes in the vascular endothelium leading to proliferative

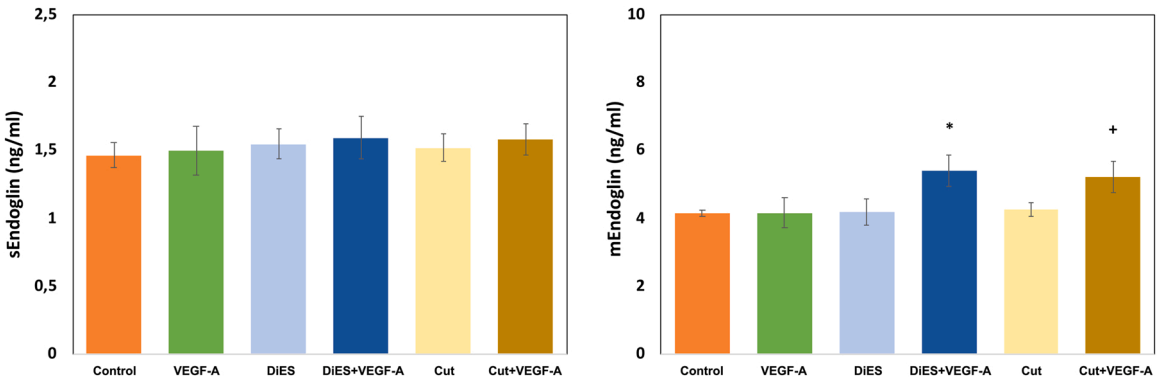

Fig. 3. Effects of DiES and Cut antigens on sEndoglin and mEndoglin in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●) for 24 h. Results are expressed as the mean ± SD of 3 independent experiments. Significant differences in comparisons with the other groups are indicated ($p < 0.05$): DiES+VEFG-A *vs* control, VEFG-A and DiES (*) and Cut+VEFG-A *vs* control, VEFG-A and Cut (+).

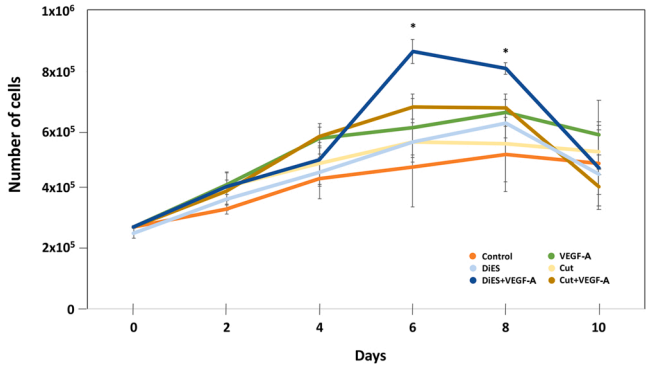

Fig. 4. Effects of DiES and Cut antigens on viable cell number in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●) every 2 days for 10 days. Results are expressed as the mean ± SD of 3 independent experiments. The asterisk (*) indicate significant differences between DiES+VEFG-A *vs* control, VEFG-A and DiES ($p < 0.05$).

endarteritis, hypertension, oedema, and pulmonary thromboembolism, among other conditions (Morchón et al., 2018, McCall et al., 2008). Previous studies have shown that both somatic antigens of *D. immitis* and recombinant *Wolbachia* Surface Major protein (rWSP) are able to promote inflammatory processes causing vasoconstriction and chemotaxis of immune cells in an *in-vitro* model of endothelial cells (Morchón et al., 2018, Simón et al., 2008), as well as a strong inflammatory response when adult worms die and antigens (both parasite and *Wolbachia*) are released in infected dogs (Morchón et al., 2012). However, DiES is able to stimulate vasodilation (Morchón et al., 2010), and to activate the fibrinolytic system, which promotes the lysis of fibrin clots (González-Miguel et al., 2012), in the same way that Cut does (González-Miguel et al., 2013). In addition, neither DiES nor Cut not contain *Wolbachia* antigens (Simón et al., 2012, Morchón et al., 2014) so DiES and Cut could be related to mechanisms that promote the survival of *D. immitis* in the vascular endothelium.

There are few studies that address the angiogenic process of heartworm disease. There are studies in which *D. immitis* adult worm somatic antigen (DiSA) promotes the production of proangiogenic molecules in endothelial cells according to the production of VEFG-A (Zueva et al., 2019) as well as the stimulation of the anti-angiogenic mechanism by *Wolbachia* and inhibition of pseudo-capillary formation (Zueva et al., 2020). On the other hand, remodeling of lymphatic and blood vessels in other filariosis has been reported. Cultures of lymphatic endothelial cells in the presence of live worms or *Wuchereria bancrofti* antigens activated lymphatic vessel remodeling, causing cell proliferation and cell differentiation. These events have been linked to processes that facilitate vascular irrigation in damaged tissues (Wysmołek et al., 1994). However, some authors have shown that angiogenesis and/or lymphangiogenesis can be induced by various factors derived from adult worm-activated monocytes (Dennis et al., 2011, Bennuru et al., 2010, Narasimhan et al., 2018).

The aim of this study was to analyze the angiogenic capacity of DiES and Cut from live adult worms in an *in-vitro* model of endothelial cells. For this purpose, an *in-vitro* endothelial cell model with HUVECs was used to recreate the angiogenic process produced by live *D. immitis* adult worm obstruction of blood vessels in dogs with heartworm. There are studies where both human and canine endothelial cells are used to carry out this type of study (Simón et al., 2008; Morchón et al., 2008; Zueva et al., 2019, 2020; Pérez Rodríguez et al., 2023) where no differences in the final result are observed.

Both DiES and Cut were derived from different adult worms, which was done to avoid possible interference from loss of molecules from the use of the same adult worms to obtain the antigens used in this study.

For the angiogenic process to be initiated, the first molecule produced is the production of VEFG-A (Karamysheva, 2008). To simulate this, stimulations with DiES and Cut from live adult worms together with VEFG-A were performed on endothelial cells and stimulation with both antigens separately. Using this endothelial cell model, the results have shown that excretory/secretory antigens and surface molecules from adult live *D. immitis* worms are able to promote the stimulation of proangiogenic factors as well as cell viable number and cell migration and the formation of pseudo-capillaries, without altering cell viability.

The effects of both antigens on VEFG-A, the main angiogenic factor, and other pro-antiangiogenic (sVEFGR-2 and mEndoglin) and anti-angiogenic (VEFGR-1/sFlt1 and sEndoglin) molecules were analysed. On the one hand, DiES and Cut +VEFG-A were able to stimulate the production of VEFG-A in endothelial cells. A similar result has been observed in human endothelial cells stimulated with DiES after being subjected or not to hypoxia, and *Wolbachia* under normoxic conditions (Simón et al., 2012, Morchón et al., 2008, Zueva et al., 2019, 2020). Furthermore, the results showed an increase in the production of sVEFGR-2, a soluble VEFG-A receptor that promotes the proangiogenic pathway, when endothelial cells were stimulated with DiES+VEFG-A and Cut+VEFG-A, a date that occurs in similar situations where vascular damage occurs (Melincovici et al., 2018, Katoh, Targeting, 2016, Peach et al., 2018). However, DiES+VEFG-A did not alter the production of VEFGR-1/sFlt1 except in the case of Cut+VEFG-A where its production was reduced, being an anti-angiogenic factor, as described in other studies (Shibuya, 2015, Zhang et al., 2002).

On the other hand, only stimulation of both DiES+VEFG-A and Cut+VEFG-A was able to cause a significant increase in the production of mEndoglin, and not sEndoglin, in the present endothelial cell model.

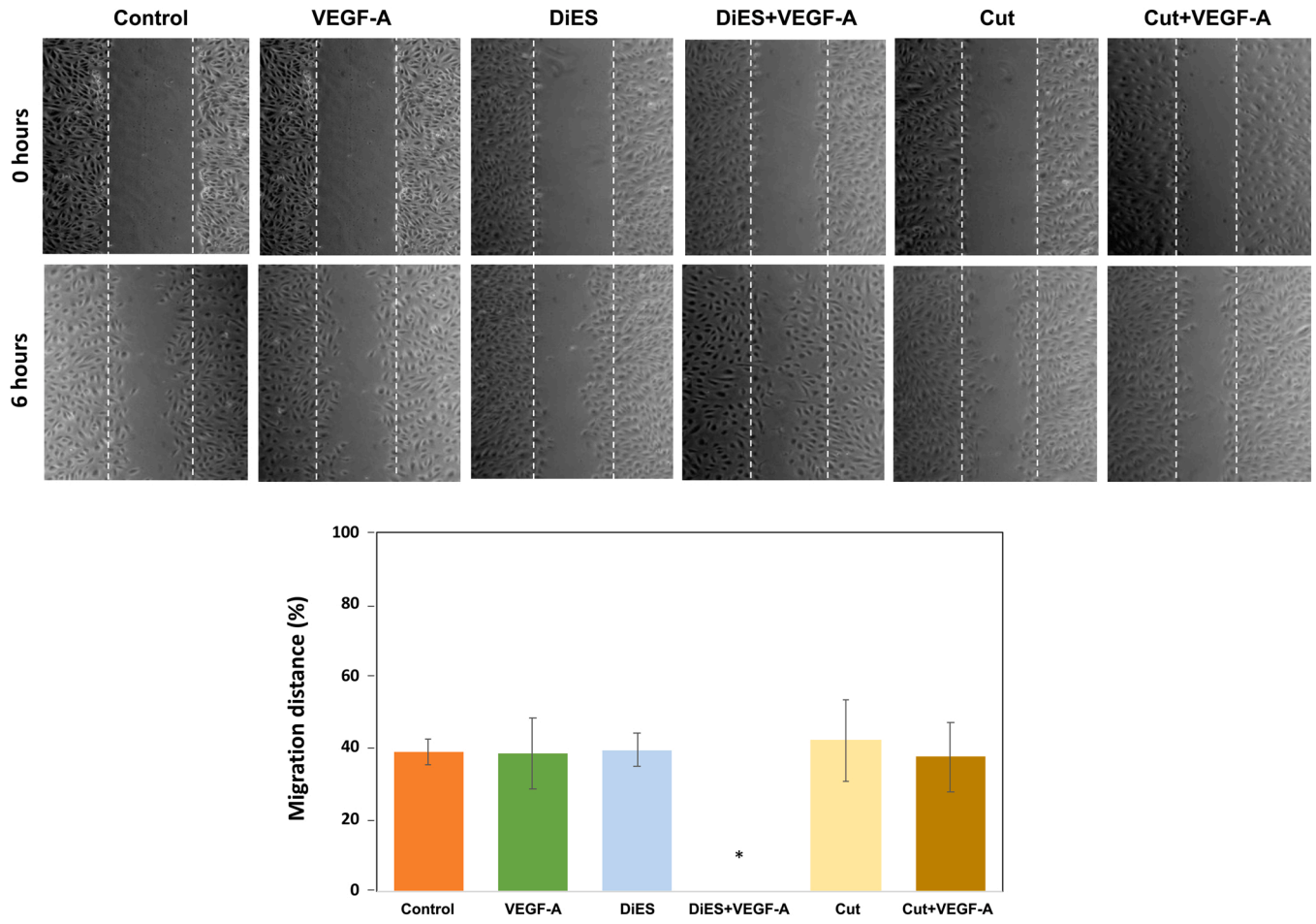

Fig. 5. Effects of DiES and Cut antigens on cell migration distance in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●). The quantification was carried out by measuring the distance of cell migration in comparison with control (untreated cells) to 6 h post-stimulation. Results are expressed as the mean ± SD of 3 independent experiments. The asterisk (*) indicate significant differences between DiES+VEFG-A *vs* control, VEFG-A and DiES ($p < 0.05$).

mEndoglin is the membrane-bound form related to proangiogenic processes, while sEndogline in the soluble form related to anti-angiogenic mechanisms and proinflammatory processes (Ollauri-Ibáñez et al., 2017). In addition, endoglin suppression in HUVECs produced the inhibition of angiogenesis *in-vitro* (Liu et al., 2014). Therefore, the present results confirm previous findings, showing that DiES and Cut together with VEFG-A are able to activate proangiogenic processes, which could facilitate the survival of adult *D. immitis* worms.

Finally, the effect of DiES and Cut on endothelial cell viability during the first 10 days was studied, as well as cell migration and pseudo-capillary formation, processes directly related to angiogenesis (Tahergorabi & Khazaei, 2012, Gerhardt et al., 2013, Hazarika et al., 2007, Liu et al., 2014). Only DiES+VEFG-A produced an increase in viable cells and stimulated cell migration, as well as the formation of pseudo-capillaries in the present endothelial cell model. The increase of VEFG-A levels after stimulation is beneficial for angiogenesis, which promotes the increase in both endothelial proliferation and migration (Mühleder et al., 2021, Byrne et al., 2005), processes involved in tumor vascularization and activation of inflammatory mechanisms (Laurenzana et al., 2015, Aguilar-Cazares et al., 2019). Moreover, the formation of pseudo-capillaries which resemble small blood vessels in formation during angiogenesis Francescone et al., 2011, Beloglazova et al., 2022), was not altered when endothelial cells were stimulated with DiSA (Zueva et al., 2019) and was even inhibited by *Wolbachia* Zueva et al., 2020), which could confirm that DiES would facilitate the survival of adult *D. immitis* worms, since, upon their death, this process is altered.

In conclusion, this is the first study to demonstrate the ability of excretory/secretory and surface-associated antigens of adult *D. immitis* worms to activate the production of some proangiogenic factors, and only excretory/secretory antigens of adult *D. immitis* worms produces an increase in viable cells and stimulate cell migration, as well as formation of pseudo-capillaries in cultured endothelial cells *in-vitro*. Considering that these processes occur in dogs with heartworm, in which adult worms produce obstructive inflammatory processes and ischemia, and that somatic antigens of *D. immitis* and *Wolbachia* promote the production of anti-angiogenic factors, the present results suggest that the excretory/secretory antigens of adult *D. immitis* worms could favor parasite survival in the vascular endothelium by stimulating proangiogenic processes.

Funding

This study was supported by Agencia de Desarrollo Económico de Castilla y León, Spain (cofinanced with FEDER funds) and General Foundation of University of Salamanca (Plan TCUE).

CRediT authorship contribution statement

C.V. Cardona Machado: Formal analysis of the data, Methodology, Writing – original draft. **C. Alarcón-Torrecillas:** Formal analysis of the data, Methodology. **M. Pericacho:** Conceptualization, Methodology,

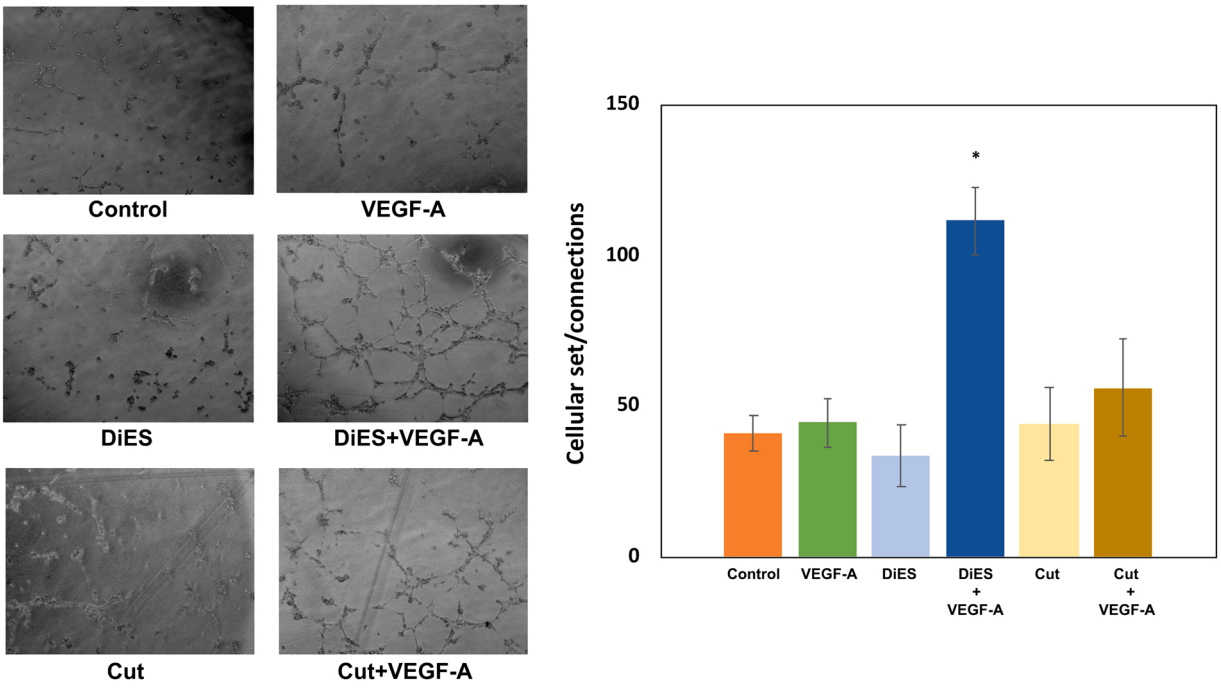

Fig. 6. Effects of DiES and Cut antigens on connections and cellular set in non-stimulated cultures (●) and cultures stimulated with VEFG-A (●), DiES (●), DiES+VEFG-A (●), Cut (●) and Cut+VEFG-A (●) for the first 3 h. Results are expressed as the mean ± SD of 3 independent experiments. The asterisk (*) indicate significant differences between DiES+VEFG-A *vs* control, VEFG-A and DiES ($p < 0.05$).

Resources, Supervision of the research. **I. Rodríguez-Escolar**: Methodology, Writing – original draft. **E. Carretón:** Methodology, Supervision of the research, Writing – original draft. **J.A. Montoya-Alonso:** Supervision of the research, Writing – original draft. **R. Morchón:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision of the research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Not applicable.

References

Aguilar-Cazares, D., Chávez-Domínguez, R., Carlos-Reyes, A., López-Camarillo, C., Hernández de la Cruz, O.N., López-González, J.S., 2019. Contribution of angiogenesis to inflammation and cancer. Front. Oncol. 9, 1399.

Ames, M.K., Atkins, C.E., 2020. Treatment of dogs with severe heartworm disease. Vet. Parasitol. 283, 109131.

Beloglazova, I., Zubkova, E., Dergilev, K., Goltseva, Y., Parfyonova, Y., 2022. New insight on 2D in-vitro angiogenesis models: all that stretches is not a tube. Cells 11 (20), 3278.

Bennuru, S., Maldarelli, G., Kumaraswami, V., Klion, A.D., Nutman, T.B., 2010. Elevated levels of plasma angiogenic factors are associated with human lymphatic filarial infections. Am. J. Trop. Med. Hyg. 83 (4), 884–890.

Betsholtz, C., 2004. Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev. 15, 215–228.

Byrne, A.M., Bouchier-Hayes, D.J., Harmey, J.H., 2005. Angiogenic and cell survival functions of vascular endothelial growth factor (VEFG-A). J. Cell. Mol. Med 9 (4), 777–794.

Dennis, R., Schubert, U., Bauer, C., 2011. Angiogenesis and parasitic helminth-associated neovascularization. Parasitol 138 (4), 426–439.

Dillon, A.R., Warner, A.E., Brawner, W., Hudson, J., Tillson, M., 2008. Activity of pulmonary intravascular macrophages in cats and dogs with and without adult Dirofilaria immitis. Vet. Parasitol. 158, 171–176.

Diosdado, A., Simón, F., Morchón, R., González-Miguel, J., 2020. Dirofilaria immitis possesses molecules with anticoagulant properties in its excretory/secretory antigens. Parasitology 147 (5), 559–565.

Eilken, H.M., Adams, R.H., 2010. Dynamics of endothelial cell behavior in sprouting angiogenesis. Curr. Opin. Cell Biol. 22, 617–625.

Francescone 3rd, R.A., Faibish, M., Shao, R., 2011. A Matrigel-based tube formation assay to assess the vasculogenic activity of tumor cells. J. Vis. Exp. 55, 3040.

Gallardo-Vara, E., Tual-Chalot, S., Botella, L.M., Arthur, H.M., Bernabeu, C., 2018. Soluble endoglin regulates expression of angiogenesis-related proteins and induction of arteriovenous malformations in a mouse model of hereditary hemorrhagic telangiectasia. Dis. Model Mech. 11 dmm034397.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., Betsholtz, C., 2003. VEFG-A guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. 161, 1163–1177.

González-Miguel, J., Morchón, R., Mellado, I., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2012. Excretory/secretory antigens from Dirofilaria immitis adult worms interact with the host fibrinolytic system involving the vascular endothelium. Mol. Biochem. Parasitol. 181, 134–140.

González-Miguel, J., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2013. Surface associated antigens of Dirofilaria immitis adult worms activate the host fibrinolytic system. Vet. Parasitol. 196 (1–2), 235–240.

González-Miguel, J., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Simón, F., 2015. Can the activation of plasminogen/plasmin system of the host by metabolic products of Dirofilaria immitis participate in heartworm disease endarteritis? Parasit. Vectors 8, 194.

Gregory, A.L., Xu, G., Sotov, V., Letarte, M., 2014. Review: the enigmatic role of endoglin in the placenta. Placenta 35, S93–S99.

Hazarika, S., Dokun, A.O., Li, Y., Popel, A.S., Kontos, C.D., Annex, B.H., 2007. Impaired angiogenesis after hindlimb ischemia in type 2 diabetes mellitus: differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. Circ. Res. 101 (9), 948–956.

Hershey, J.C., Baskin, E.P., Glass, J.D., Hartman, H.A., Gilberto, D.B., Rogers, I.T., Cook, J.J., 2001. Revascularization in the rabbit hindlimb: dissociation between capillary sprouting and arteriogenesis. Cardiovasc. Res 49, 618–625.

Jerckic, M., Rivas-Elena, J.V., Santibáñez, J.F., Prieto, M., Rodríguez-Barbero, A., Pérez-Barriocanal, F., Pericacho, M., Arévalo, M., Vary, C.P.H., Letarte, M., Bernabeu, C., López-Novoa, J.M., 2006. Endoglin regulates cyclooxygenase-2 expression and activity. Circ. Res. 99, 248–256.

Karamysheva, A.F., 2008. Mechanisms of angiogenesis. Biochemistry (Mosc.) 73 (7), 751–762.

Katoh, M.Therapeutics, Targeting, F.G.F., 2016. Signaling network in human diseases. Trends Pharmacol. Sci. 37 (12), 1081–1096.

Laurenzana, A., Fibbi, G., Margheri, F., Biagioni, A., Luciani, C., Del Rosso, D., Chillà, A., 2015. Endothelial progenitor cells in sprouting angiogenesis: proteases pave the way. Curr. Mol. Med. 15 (7), 606–620.

Liu, Z., Lebrin, F., Maring, J.A., van den Driesche, S., van der Brink, S., van Dinther, M., et al., 2014. Endoglin is dispensable for vasculogenesis, but required for vascular endothelial growth factor-induced angiogenesis. PloS One 9, e86273.
McCall, J.W., Genchi, C., Kramer, L.H., Guerrero, J., Venco, L., 2008. Heartworm disease in animals and humans. Adv. Parasitol. 66, 193–285.
Melincovici, C.S., Boşca, A.B., Şuşman, S., Mărginean, M., Mihu, C., Istrate, M., Moldovan, I.M., Roman, Al, Mihu, C.M., 2018. Vascular endothelial growth factor (VEFG-A) - key factor in normal and pathological angiogenesis. Rom. J. Morphol. Embryol. 59 (2), 455–467.
Morchón, R., Carretón, E., Grandi, G., González-Miguel, J., Montoya-Alonso, J.A., Simón, F., Genchi, C., Kramer, L.H., 2012. Anti-Wolbachia Surface Protein antibodies are present in the urine of dogs naturally infected with Dirofilaria immitis with circulating microfilariae but not in dogs with occult infections. Vector Borne Zoonotic Dis. 12 (1), 17–20.
Morchón, R., González-Miguel, J., Mellado, I., Velasco, S., Rodríguez-Barbero, A., Simón, F., 2010. Adult Dirofilaria immitis excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration in an "in-vitro" model of vascular endothelial cell cultures. Vet. Parasitol. 170, 331–335.
Morchón, R., González-Miguel, J., Carretón, E., Kramer, L.H., Valero, L., Montoya-Alonso, J.A., Simón, F., Siles-Lucas, M., 2014. Proteomic analysis of the somatic and surface compartments from Dirofilaria immitis adult worms. Vet. Parasitol. 203 (1–2), 144–152.
Morchón, R., Montoya-Alonso, J.A., Rodríguez-Escolar, I., Carretón, E., 2022. What has happened to heartworm disease in Europe in the last 10 years? Pathogens 11, 1042.
Morchón, R., Rodríguez-Barbero, A., Velasco, S., López-Belmonte, J., Simón, F., 2008. Vascular endothelial cell activation by adult Dirofilaria immitis antigens. Parasitol. Int. 57, 441–446.
Mühleder, S., Fernández-Chacón, M., Garcia-Gonzalez, I., Benedito, R., 2021. Endothelial sprouting, proliferation, or senescence: tipping the balance from physiology to pathology. Cell Mol. Life Sci. 78 (4), 1329–1354.
Narasimhan, P.B., Akabas, L., Tariq, S., Huda, N., Bennuru, S., Sabzevari, H., Hofmeister, R., Nutman, T.B., Tolouei Semnani, R., 2018. Similarities and differences between helminth parasites and cancer cell lines in shaping human monocytes: Insights into parallel mechanisms of immune evasion. PLoS Negl. Trop. Dis. 12 (4), e0006404.
Ollauri-Ibáñez, C., López-Novoa, J.M., Pericacho, M., 2017. Endoglin-based biological therapy in the treatment of angiogenesis-dependent pathologies. Expert. Opin. Biol. Ther. 17 (9), 1053–1063.
Peach, C.J., Mignone, V.W., Arruda, M.A., Alcobia, D.C., Hill, S.J., Kilpatrick, L.E., Woolard, J., 2018. Molecular pharmacology of VEFG-A isoforms: binding and signalling at VEFG-AR2. Int. J. Mol. Sci. 19 (4), 1264.
Pérez Rodríguez, M.D.P., Alarcón-Torrecillas, C., Pericacho, M., Rodríguez-Escolar, I., Carretón, E., Morchón, R., 2023. Effect of somatic antigens of Dirofilaria repens adult worms on angiogenesis, cell proliferation and migration and pseudo-capillary formation in human endothelial cells. Parasit. Vectors 16 (1), 105.
Phng, L.K., Gerhardt, H., 2009. Angiogenesis: a team effort coordinated by notch. Dev. Cell. 16 (2), 196–208.
Shibuya, M., 2015. VEFG-A-VEFG-AR system as a target for suppressing inflammation and other diseases. Endocr. Metab. Immune Disord. Drug Targets 15 (2), 135–144.
Simón, F., Morchón, R., Rodríguez-Barbero, A., López-Belmonte, J., Grandi, G., Genchi, C., 2008. Dirofilaria immitis and Wolbachia-derived antigens: its effect on endothelial mammal cells. Vet. Parasitol. 158 (3), 223–231.
Simón, F., Siles-Lucas, M., Morchón, R., González-Miguel, J., Mellado, I., Carretón, E., Montoya-Alonso, J.A., 2012. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin. Microbiol. Rev. 25, 507–544.
Tahergorabi, Z., Khazaei, M., 2012. A review on angiogenesis and its assays. Iran. J. Basic Med. Sci. 15 (6), 1110–1126.
Van, R.N., Piek, J.J., Buschmann, I., Hoefer, I., Voskuil, M., Schaper, W., 2001. Stimulation of arteriogenesis; a new concept for the treatment of arterial occlusive disease. Cardiovasc. Res. 49, 543–553.
Varejckova, M., Gallardo-Vara, E., Vicen, M., Vitverova, B., Fikrova, P., Dolezelova, E., Rathouska, J., Prasnicka, A., Blazickova, K., Micuda, S., Bernabeu, C., Nemeckova, I., Nachtigal, P., 2017. Soluble endoglin modulates the pro-inflammatory mediators NF-κB and IL-6 in cultured human endothelial cells. Life Sci. 175, 52–60.
Wedrychowicz, H., Holmes, P.H., Bairden, K., Tait, A., 1994. Surface and excretory/secretory antigens of fourth-stage larvae and adult Ostertagia circumcincta. Vet. Parasitol. 53, 117–132.
Zhang, W., Ran, S., Sambade, M., Huang, X., Thorpe, P., 2002. A monoclonal antibody that blocks VEFG-A binding to VEFG-AR2 (KDR/Flk-1) inhibits vascular expression of Flk-1 and tumor growth in an orthotopic human breast cancer model. Angiogenesis 5 (1–2), 35–44.
Zueva, T., Morchón, R., Carretón, E., Montoya-Alonso, J.A., Santana, A., Bargues, M.D., Mas-Coma, S., Rodríguez-Barbero, A., Simón, F., 2019. Angiogenic response in an in-vitro model of dog microvascular endothelial cells stimulated with antigenic extracts from Dirofilaria immitis adult worms. Parasit. Vectors 12, 315.
Zueva, T., Morchón, R., Carretón, E., Ollauri-Ibáñez, C., Pericacho, M., Rodríguez Barbero, A., Simón, F., 2020. Angiogenesis in cardiopulmonary dirofilariosis: does the Wolbachia surface protein have a pro- or anti-angiogenic effect? J. Helminthol. 94, e162.