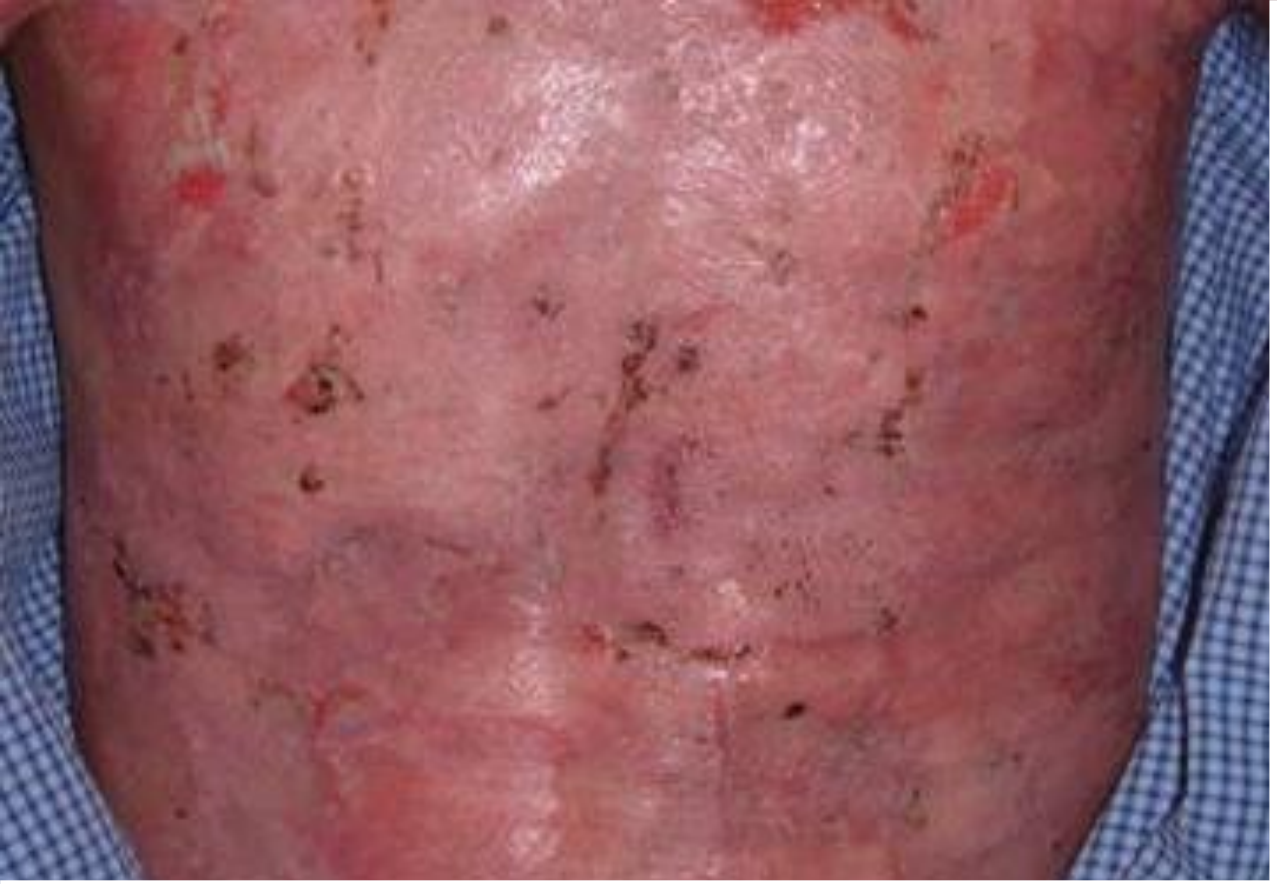
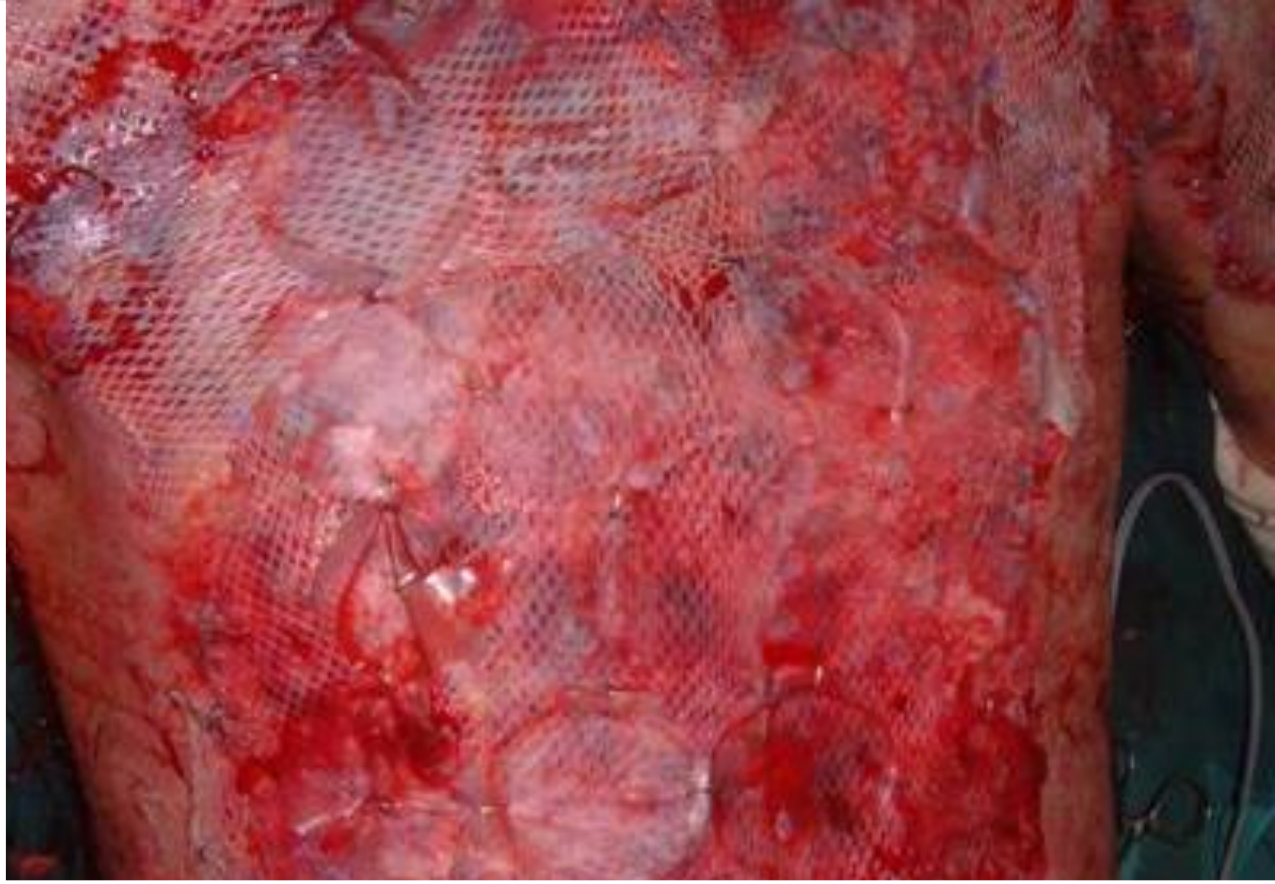
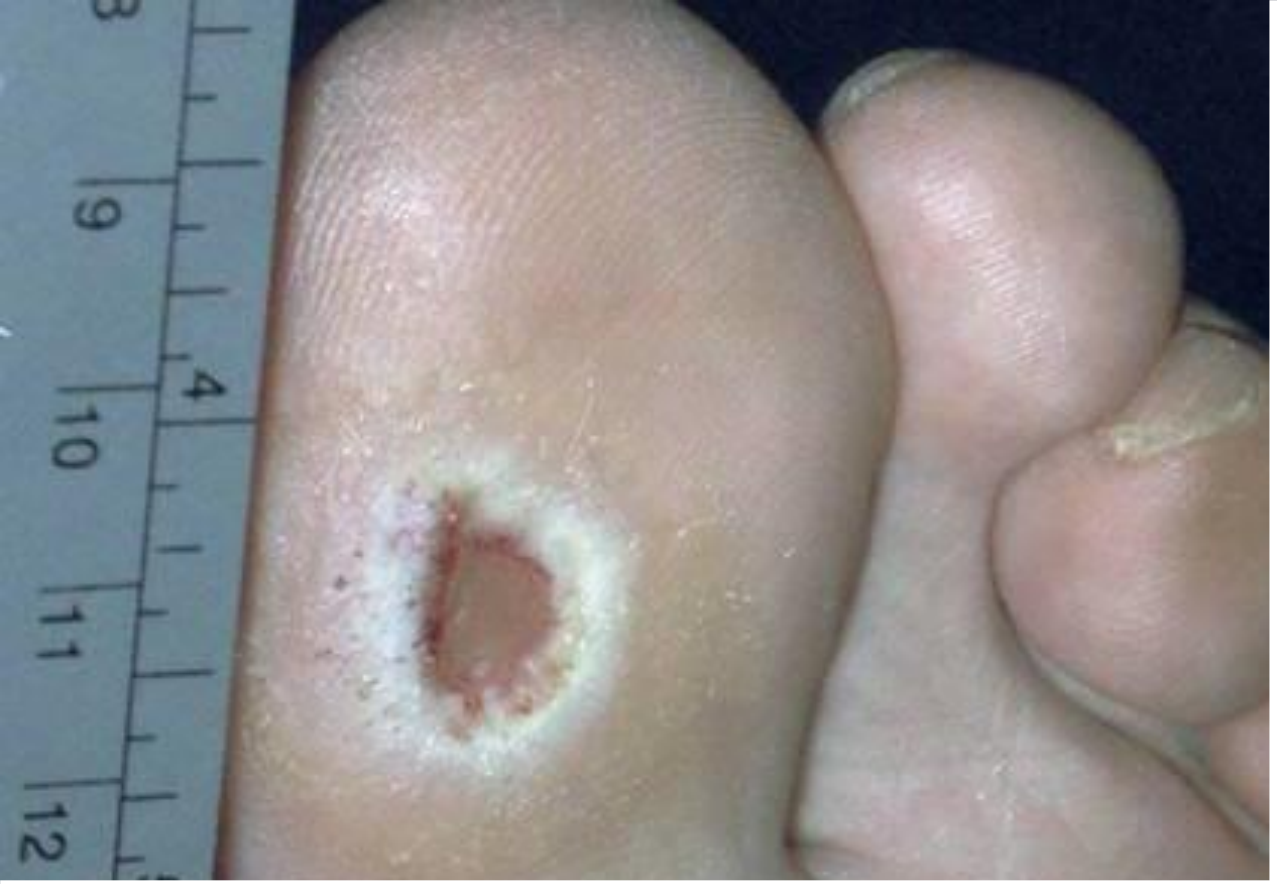
Working well together –developing interdisciplinary research that can translate to the clinic

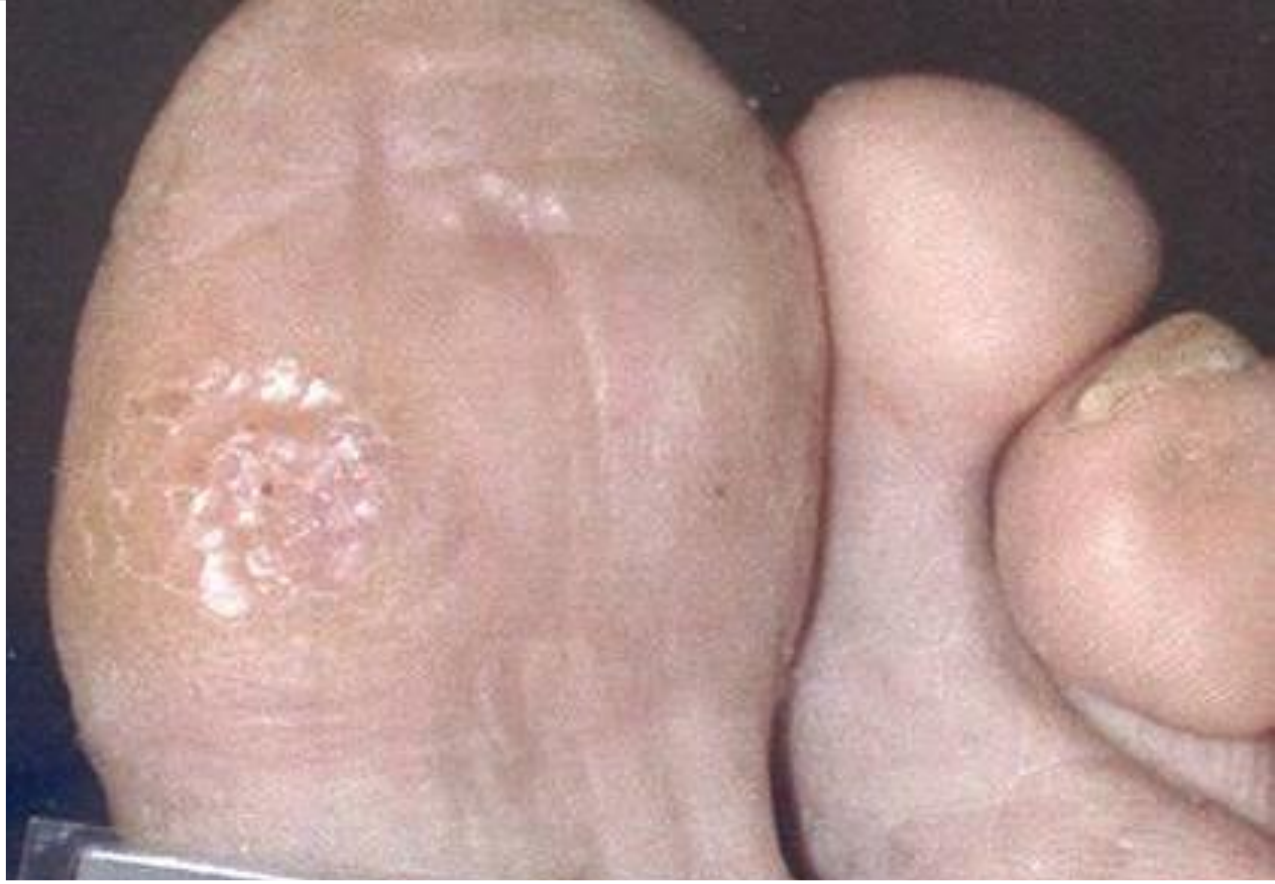
Sheila MacNeil, Muhammad Yar, Serkan Dikici and Anthony Bullock,Mariam Azam, Aqif Anwar Chaudry





Use of Myskin in acute burns – placed over wide-meshed autograft





Use of Myskin in chronic diabetic ulcers: Non-healing for 3 years and after 8 applications

AFFORDABLE HEALTHCARE FOR PAKISTAN

- Surgeons treating patients with burns do not have access to advanced wound dressings or tissue engineering therapies
- Patients with chronic non-healing ulcers also need more advanced dressings
- High technology solutions such as tissue engineering not appropriate for non healing leg ulcers and superficial burns in Pakistan
- However development of a dressing to drive angiogenesis can benefit both categories of patients

Contents of presentation

- A history of our working on 2dDR
- Does it work?
- How does it compare to VEGF in its activity and potency?
- How does it work- how much do we understand it?
- Our plans to move this to clinical benefit in Pakistan

A HISTORY OF OUR COLLABORATION

- Dec 2012 University of Sheffield Staff visit Pakistan
- August 2014 interviewed for research fellows
- November 2014 started publishing together on heparin work
- December 2015) started work on the patent
    - **a.** UK Patent KLT/P224148GB; Filing date: March 2017
    - **b.** PCT Patent PCT/GB2018/050579 Filing date:March 2018
    - **c.** 133/2018, Filling date_ March 2018
- 2016 first animal experiments
- 2018 patent licenced to Cannenta
- 2019 Cotton Craft signed research agreement with COMSATS

**Thurston G. et al. Science, 286(5449), 2511-2514, 1999*

Sheffield staff visit the IRCB in 2012 and 5 IRCB fellows visit Sheffield in 2014

Pakistani surgeons and companies sharing their experiences

Visiting the IRCBM, Comsats University, Pakistan 2019

Visiting Cotton Craft Pvt Ltd, Pakistan 2019


❖ Developing commercialisation route for sustained production and delivery of therapy
❖ Larger clinical study
❖ First in man safety study
❖ Obtaining regulatory approval
❖ Testing of materials in relevant animal models
❖ Designing and producing biomaterials
❖ Understanding the patient's needs

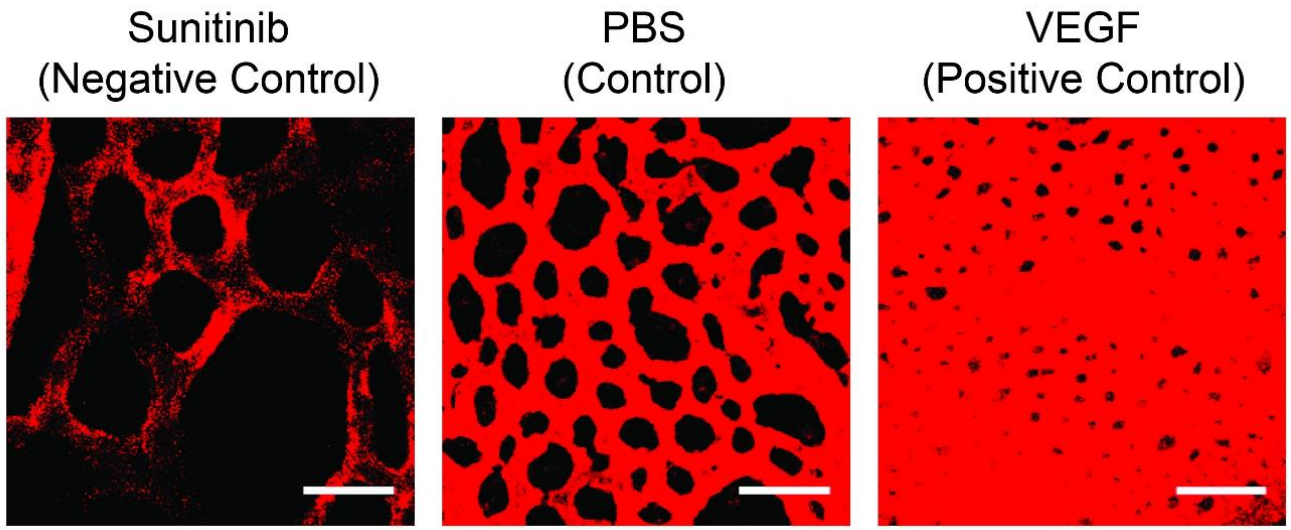
Vascularisation:

Is required for wound healing and for the translation of tissue-engineered constructs to the clinic

oxygen & nutrients must be supplied to cells upon implantation of the tissue-engineered construct

↓

Vascular Endothelial Growth Factor (VEGF)



WHY AN ALTERNATIVE TO VEGF IS NEEDED

WHY?

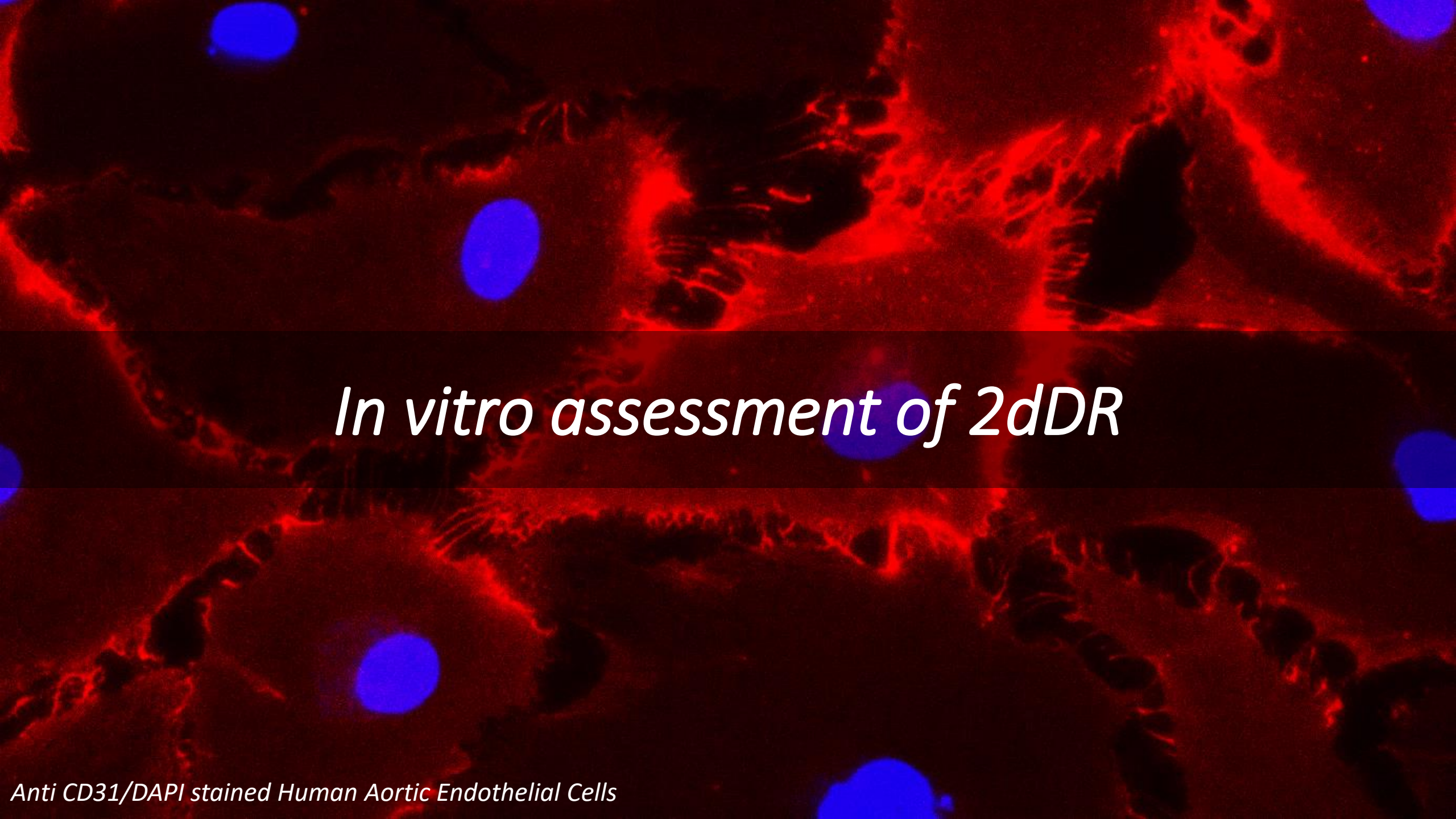
Angiongenic concentration of each drug in 1L solution

costs:

- **2dDR: ~ £0.08** (£)
- **VEGF: ~ £2000** (25000x£)

- Expensive
- Stable for 2-7 days at 2-8˚C as solution
- Uncontrolled release might lead excessively leaky, permeable and haemorrhagic vessels*

**Thurston G. et al. Science, 286(5449), 2511-2514, 1999*



In vitro assessment of 2dDR

Anti CD31/DAPI stained Human Aortic Endothelial Cells

The University Of Sheffield.

Validation of drug concentrations on HAECs in 2D

- **Metabolic Activity**
- **Cell proliferation**
- **CD31 expression**
- **Migration**
- **Tube Formation**

HAECs: *Human Aortic Endothelial Cells*

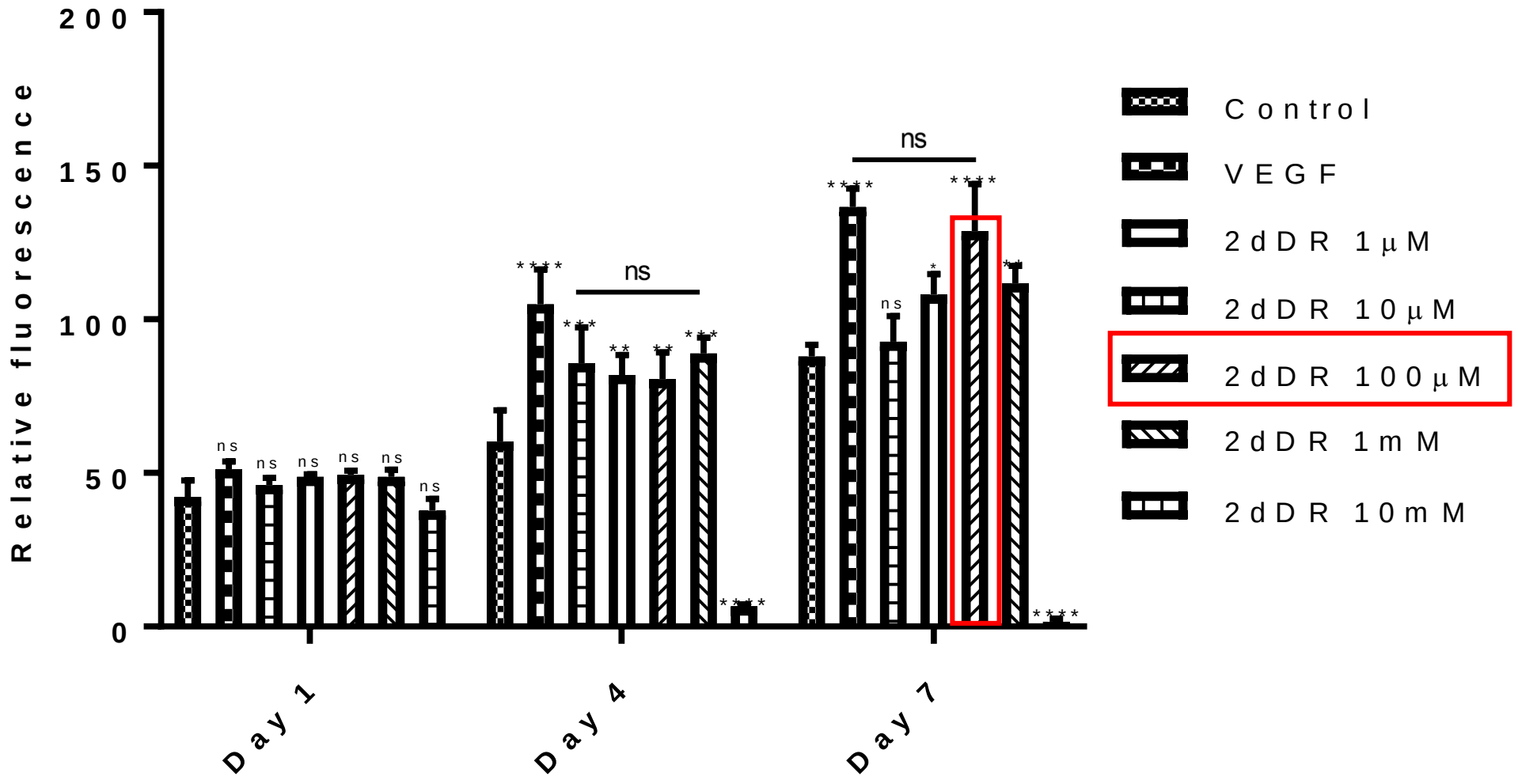
The University Of Sheffield.

Validation of drug concentrations on HAECs in 2D

- **Metabolic Activity**
- **Cell proliferation**
- **CD31 expression**
- Migration
- Tube Formation

HAECs: *Human Aortic Endothelial Cells*

AlamarBlue® Metabolic Activity (Day 1-7)

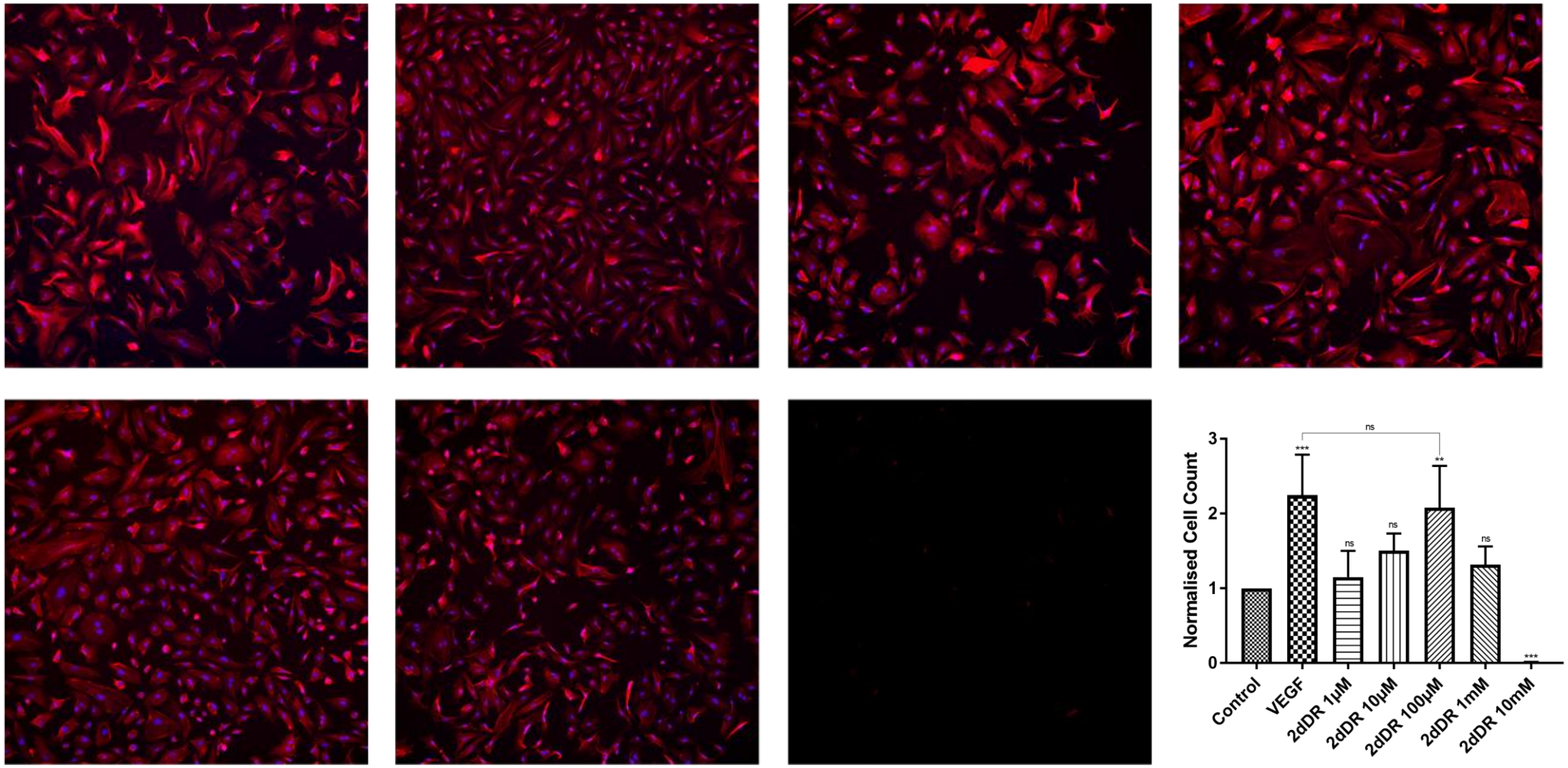


HAECs: *Human Aortic Endothelial Cells*

Alexa Fluor® 594 phalloidin / DAPI

The University Of Sheffield.

Control | VEGF | 2dDR 1µM | 2dDR 10µM

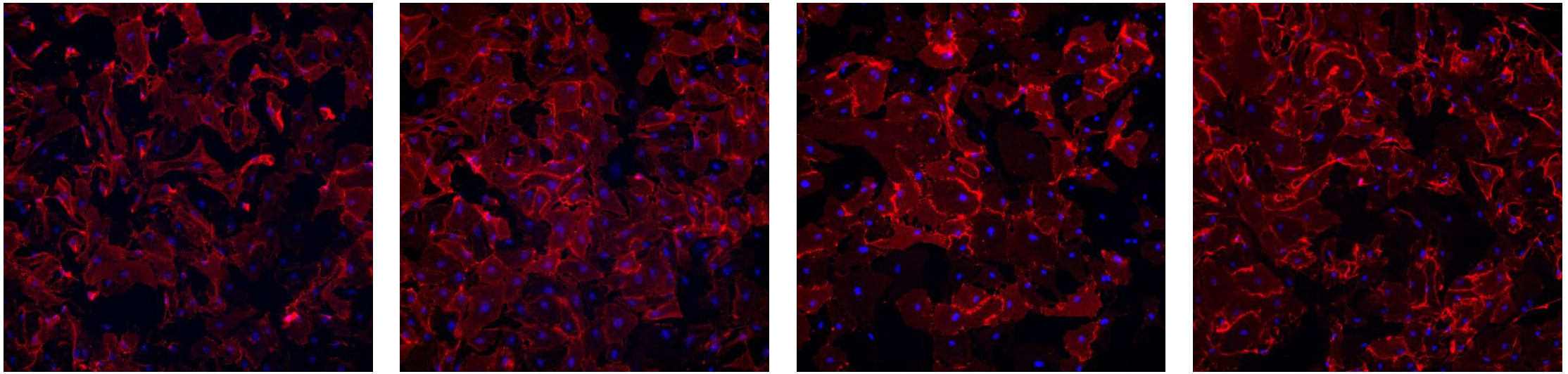


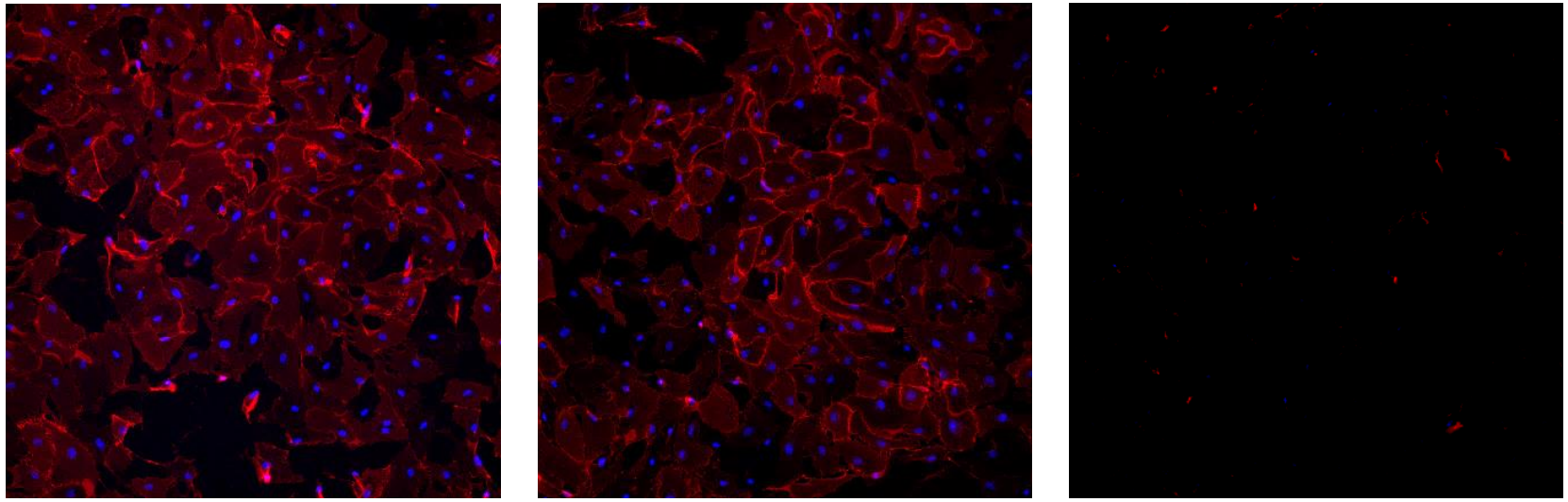
2dDR 100µM | 2dDR 1mM | 2dDR 10mM

Anti-human CD31 - IF

The University Of Sheffield.

Control **VEGF** **2dDR 1µM** **2dDR 10µM**

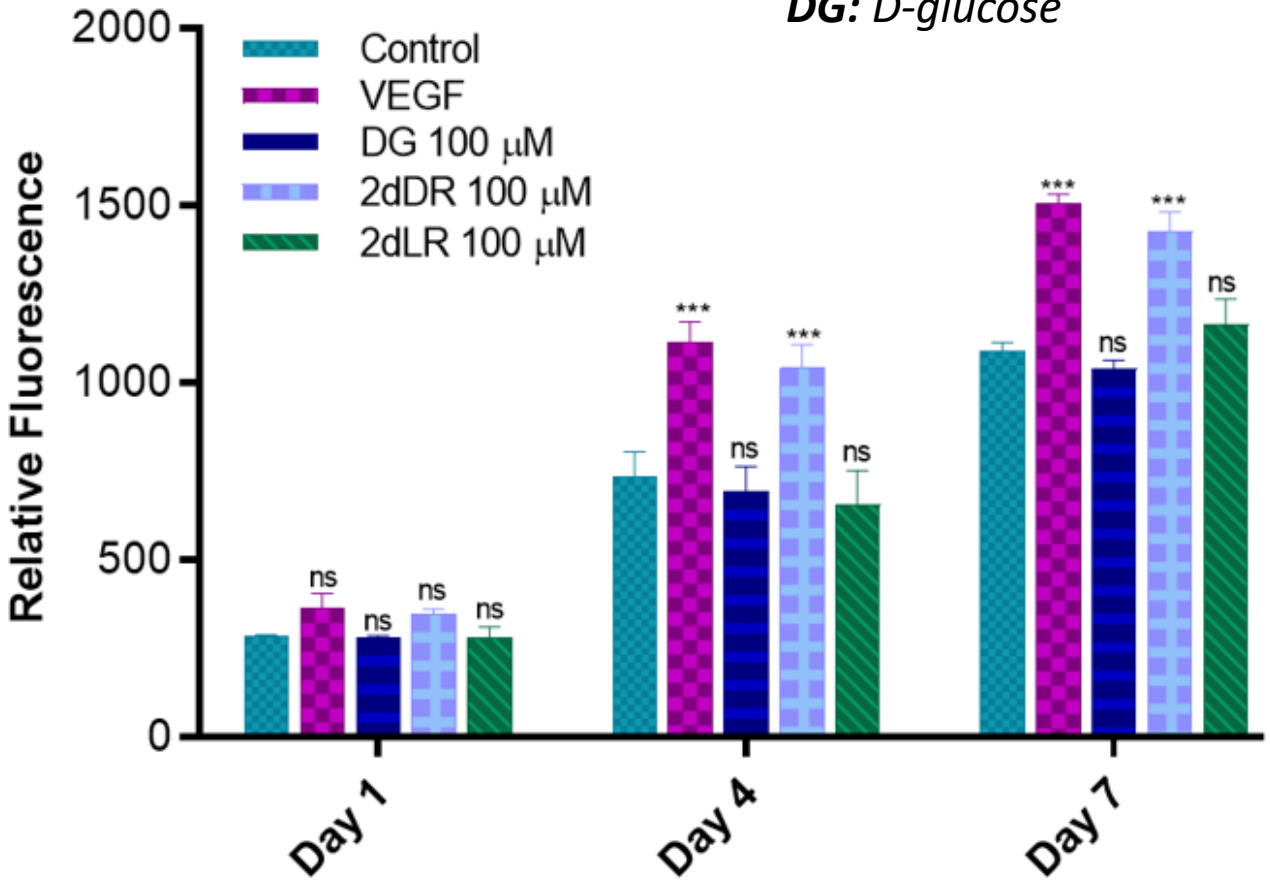




2dDR 100µM **2dDR 1mM** **2dDR 10mM**

Comparison with other sugars (Metabolic activity of HAECs)

2dDR: *2-deoxy-D-ribose*
2dLR: *2-deoxy-L-ribose*
DG: *D-glucose*



	Day 1	Day 4	Day 7
VEGF vs 2dDR	ns	ns	ns
VEGF vs 2dLR	ns	***	***
VEGF vs DG	ns	***	***
2dDR vs 2dLR	ns	***	***
2dDR vs DG	ns	***	***
2dLR vs DG	ns	ns	*

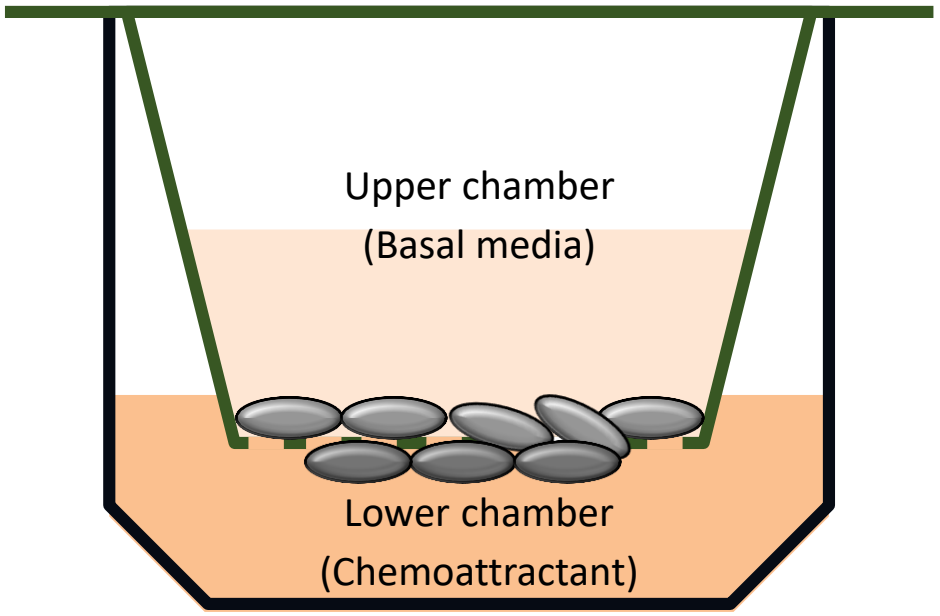
Figure *Comparison of 2dDR with other sugars (2dLR, DG, and 2dDG) in terms of increasing the metabolic activity of HAECs over 7 days in comparison with VEGF and controls. (***p ≤ 0.001, *p ≤ 0.05, not significant (ns) p ≥ 0.05, n = 3). The statistical comparison of the results is given in the table on the right.*

Validation of drug concentrations on HAECs in 2D

- Metabolic Activity
- Cell proliferation
- CD31 expression
- **Migration**
- Tube Formation

HAECs: *Human Aortic Endothelial Cells*

Boyden Chamber Migration Assay (2dDR Concentrations)



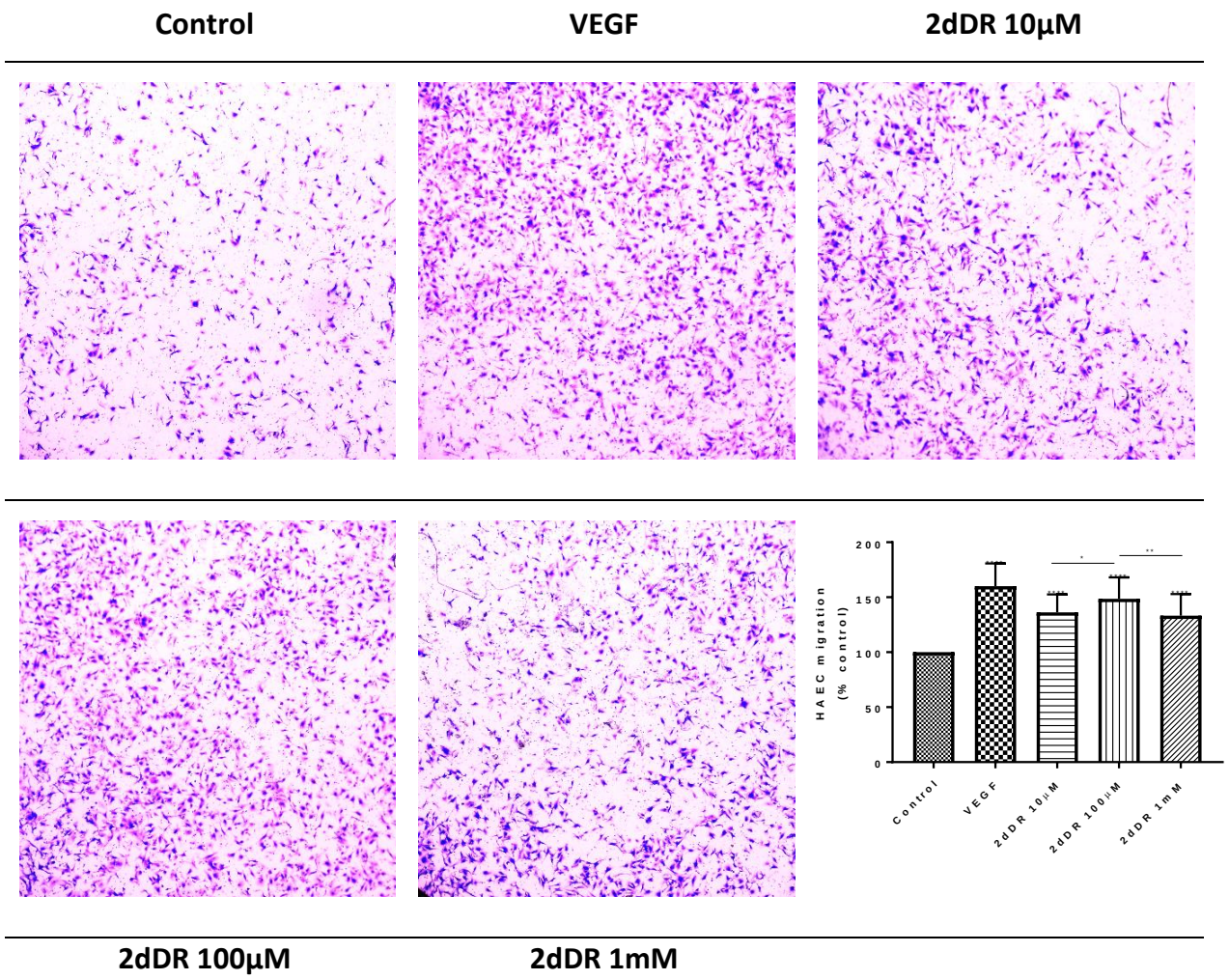


Figure *The migratory effect of different concentrations of 2dDR in comparison with VEGF and controls was evaluated by using a modified Boyden chamber assay. The quantified results were given in the graph bottom-right (***p≤0.001, **p ≤ 0.01, *p ≤ 0.05, not significant (ns) p ≥ 0.05, n = 3). Scale bars represent 250 µm.*

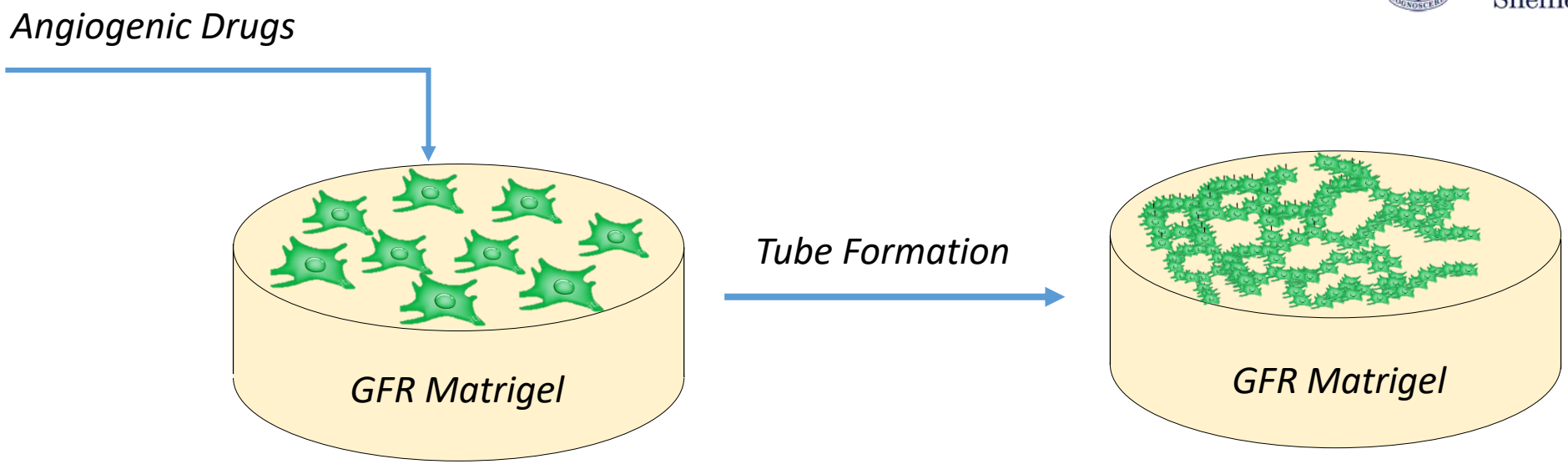
The University Of Sheffield.

Validation of drug concentrations on HAECs in 2D

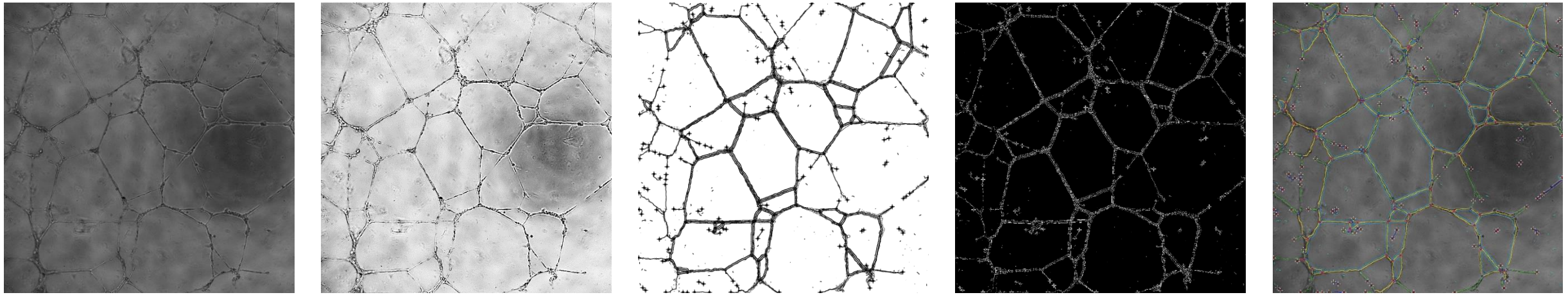
Metabolic Activity → Cell proliferation → CD31 expression → Migration → **Tube Formation**

HAECs: *Human Aortic Endothelial Cells*

Matrigel® Tube Formation Assay



Raw Image | Brightness/Contrast | Binary image | Skeletonised image | Analysed image



Matrigel® Tube Formation Assay

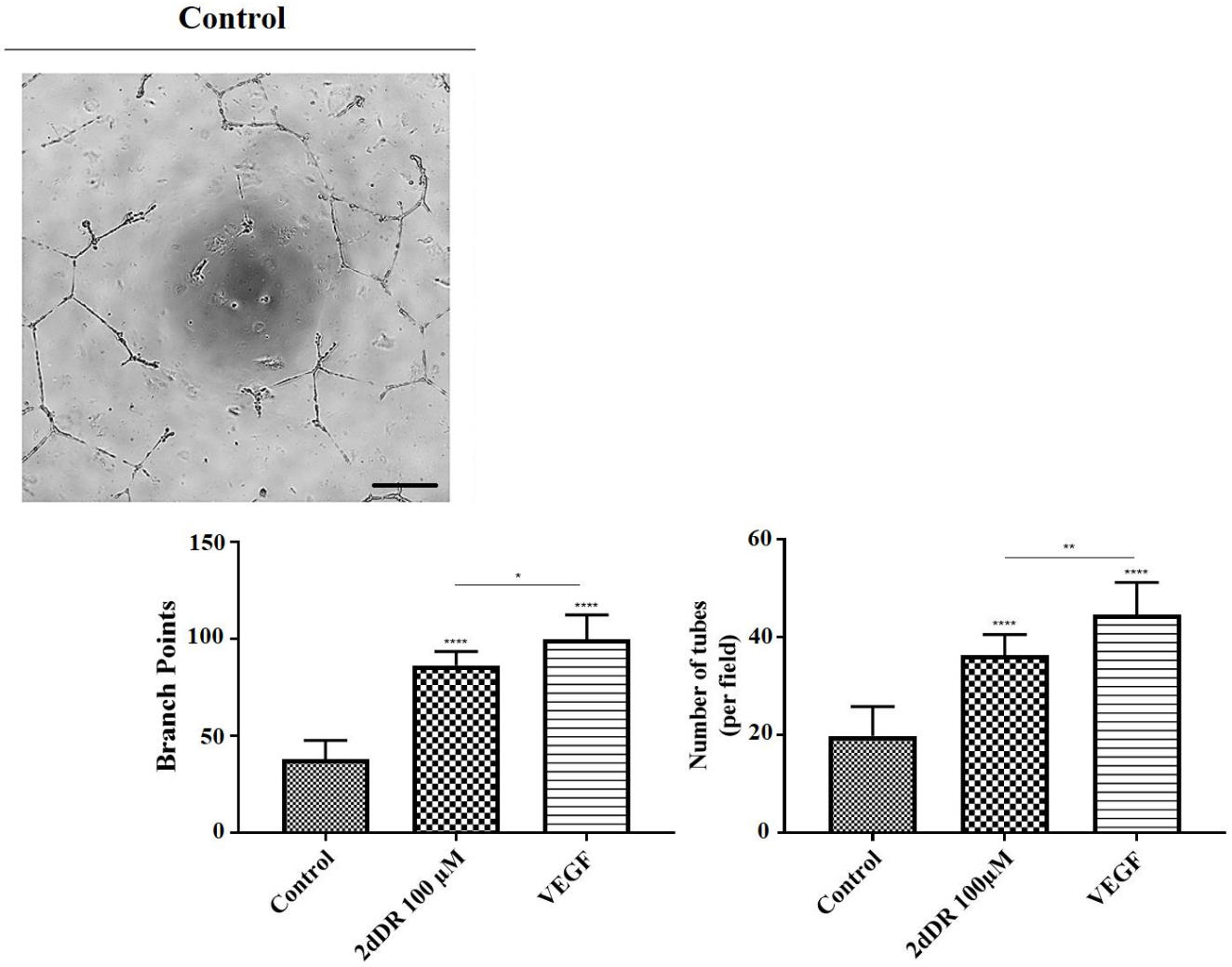
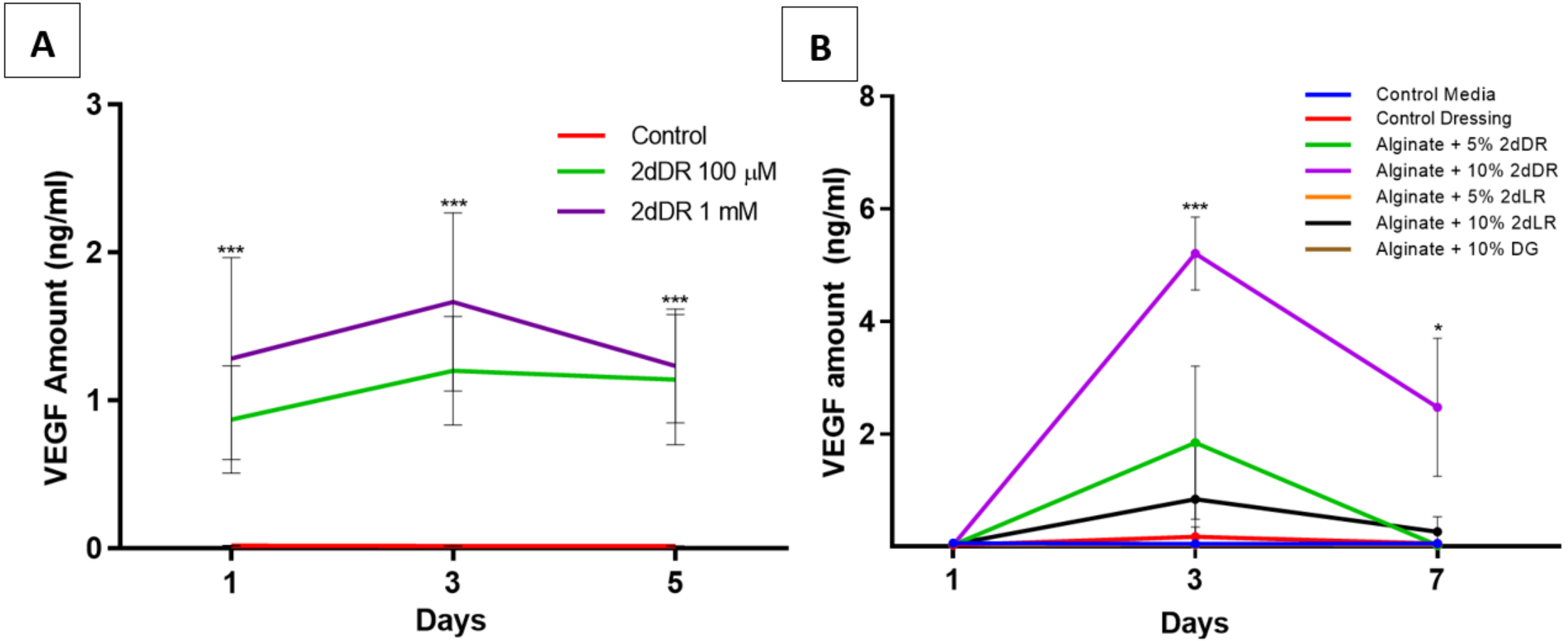


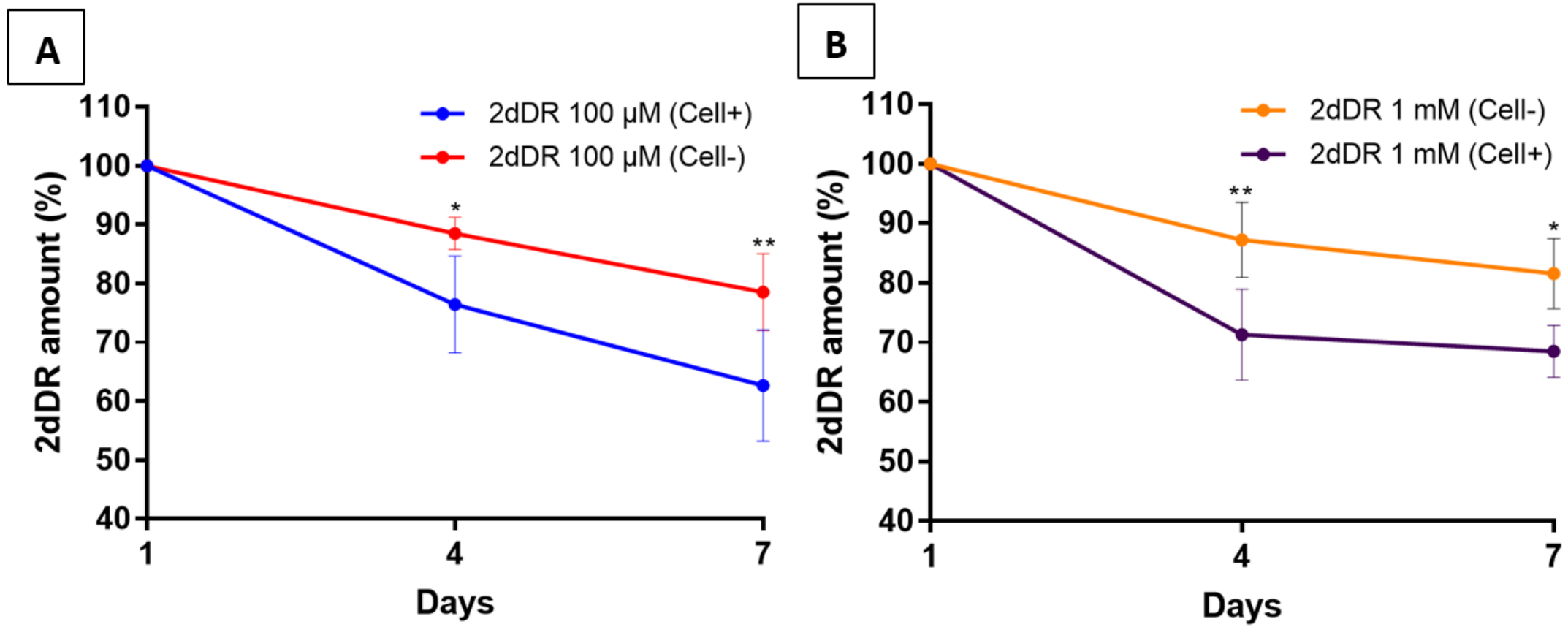
Figure *The effect of 100 µM 2dDR and VEGF on tube formation was assessed with Matrigel® tube formation assay. The quantified results of the average number of branch points and the number of tubes per field were given in the graphs given (***p≤0.001, **p ≤ 0.01, *p ≤ 0.05, not significant (ns) p ≥ 0.05, n = 3). Scale bars represent 250 µm.*

2dDR increases VEGF production by HAECs



*A .Quantification of VEGF production by HAECs in response to direct 2dDR treatment (100 µM and 1 mM). (B) VEGF production of HAECs when B .2dDR, 2dLR, and DG were released from alginate dressings. (***p ≤ 0.001, **p ≤ 0.01, *p ≤ 0.05, not significant (ns) p ≥ 0.05, n = 3).*

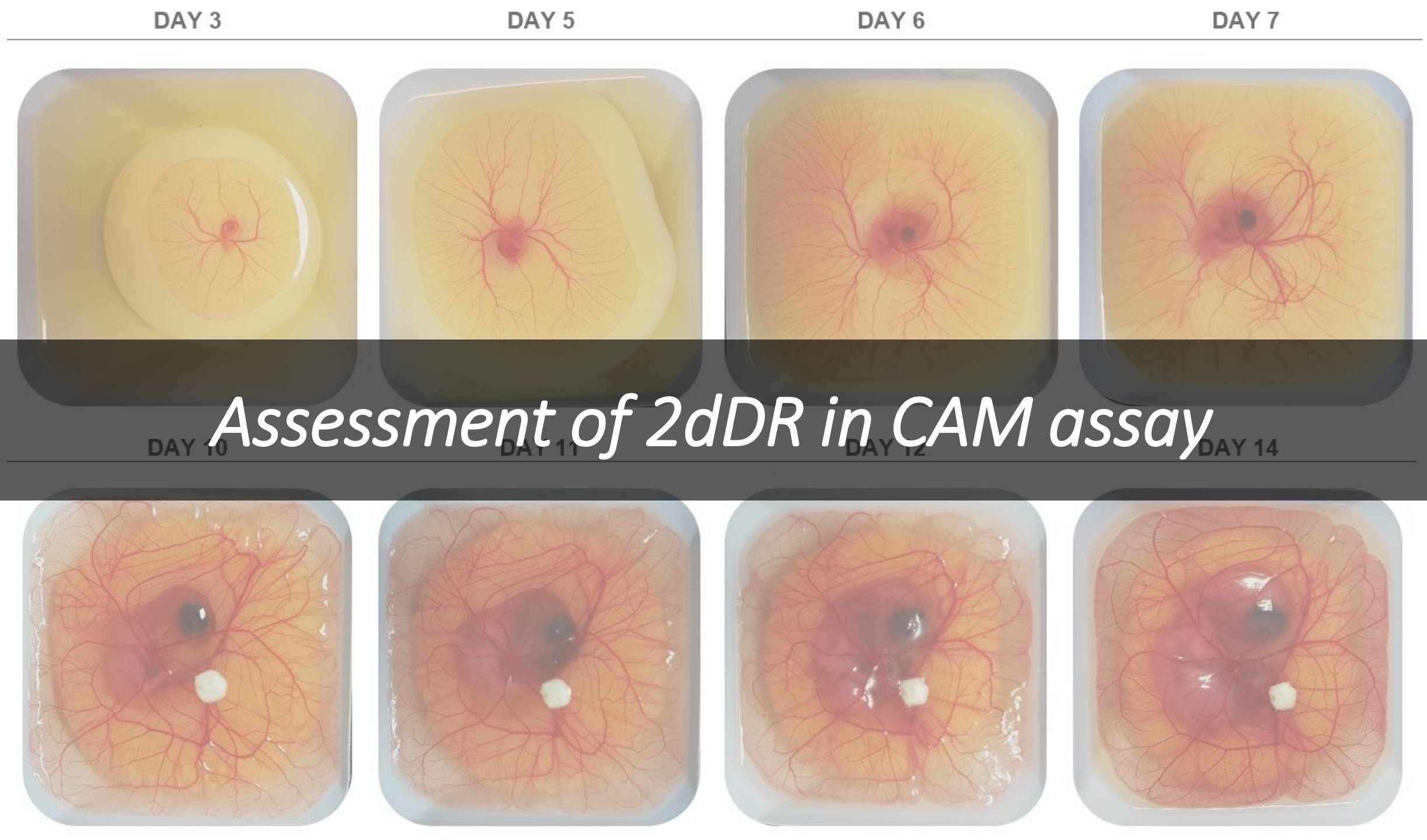
2dDR is metabolised in the presence and absence of HAECs



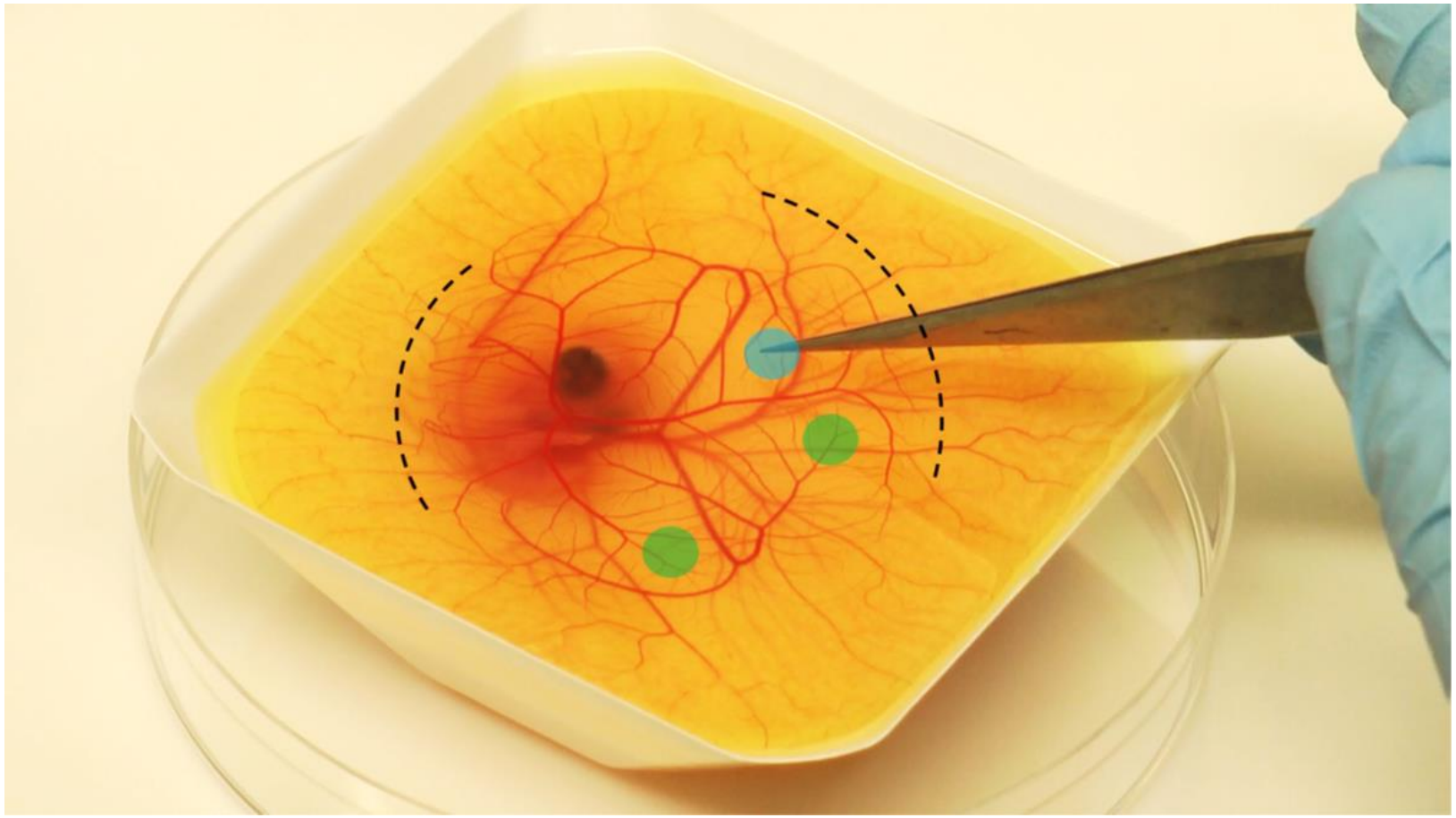
*Bial's Orcinol Assay for the assessment of the stability of 2dDR. (A) 100 µM 2dDR and (B) 1 mM 2dDR in the presence or absence of HAECs. (**p ≤ 0.01, *p ≤ 0.05, n = 3).*

Assessment of 2dDR in chick bioassay

Anti CD31/DAPI stained Human Aortic Endothelial Cells



Assessment of 2dDR in CAM assay



Chick chorioallantoic membrane (CAM):

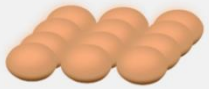
A membrane that is **rich in blood vessels** and functions as an organ for **gas exchange** between the embryo and the environment.

Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

Start of incubation

Day 0

Egg fertilisation



Start of ex-ovo culture

Day 3

Transferring the embryos into petri dishes

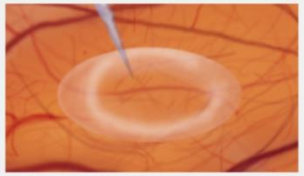
The procedure varies depending on application

Day 7

Application A

Evaluation of the angiogenic potential of different doses of drugs

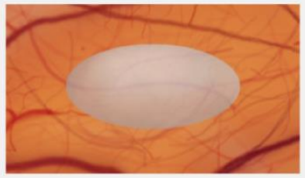
Daily injection of drugs between Day 7 and Day 11.



Application B

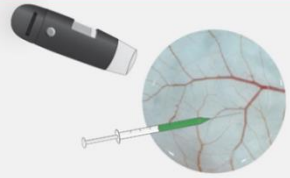
Evaluation of angiogenic potential of drug releasing scaffolds

Implantation of the drug releasing disc shaped scaffolds at Day 7.



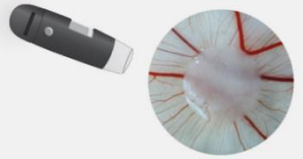
Analysis of the results

Day 11 (for Application A)



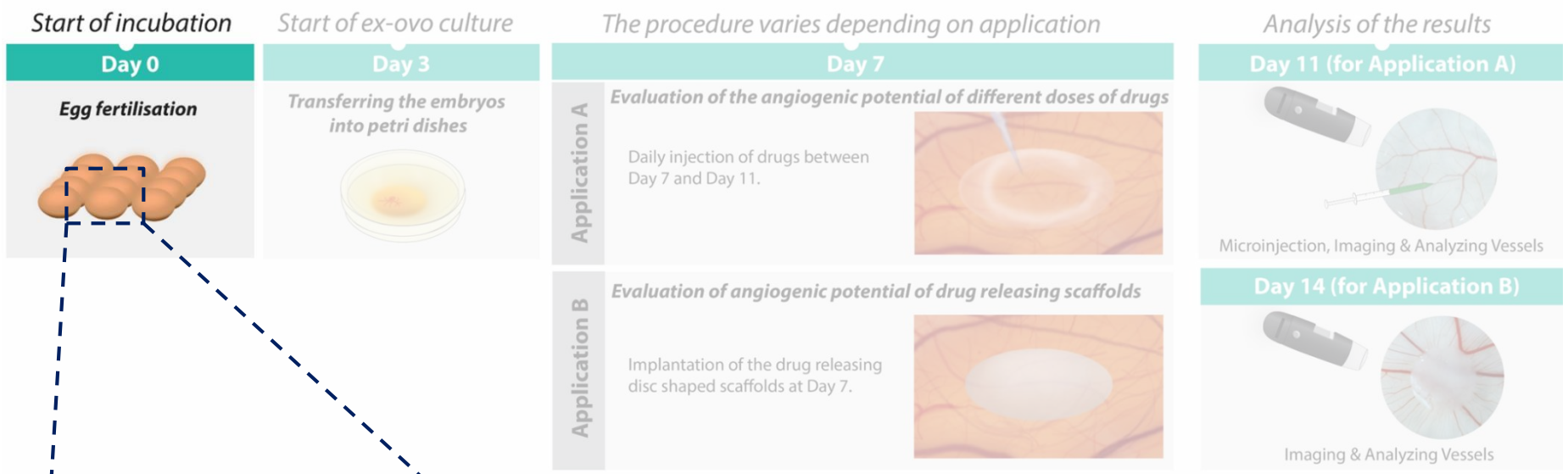
Microinjection, Imaging & Analyzing Vessels

Day 14 (for Application B)

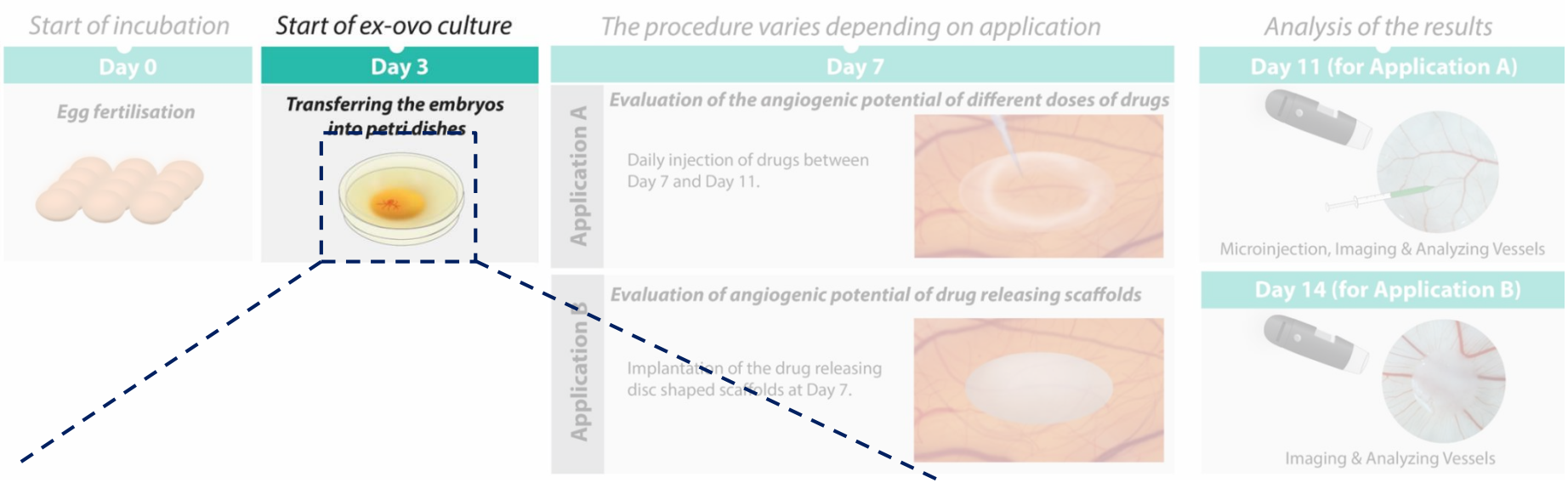


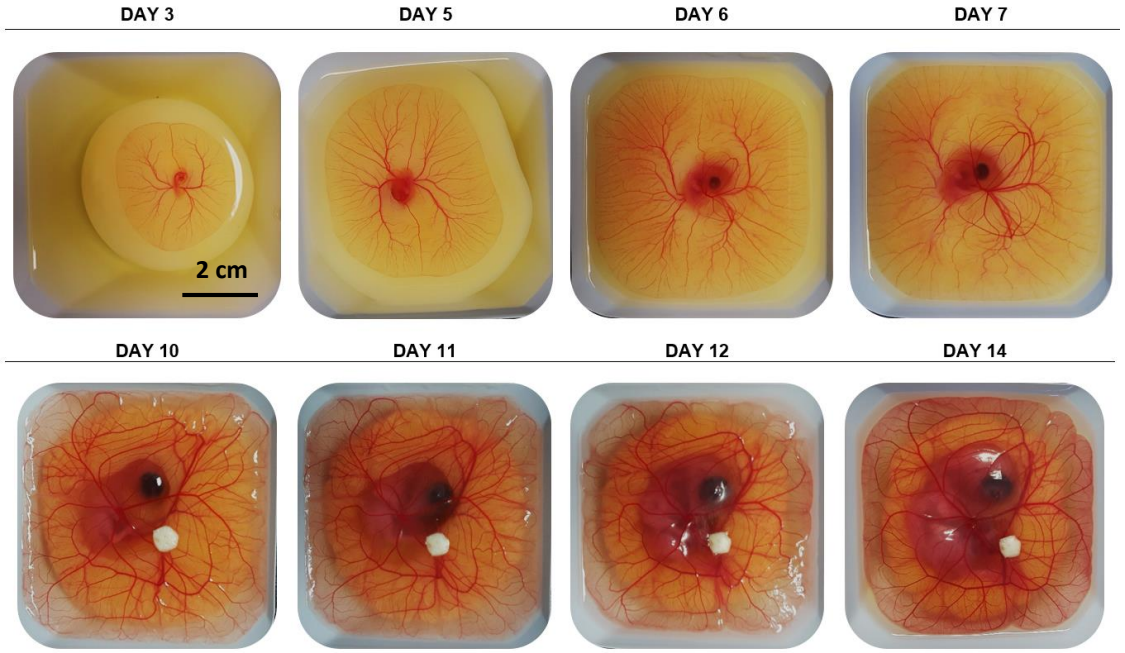
Imaging & Analyzing Vessels

Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

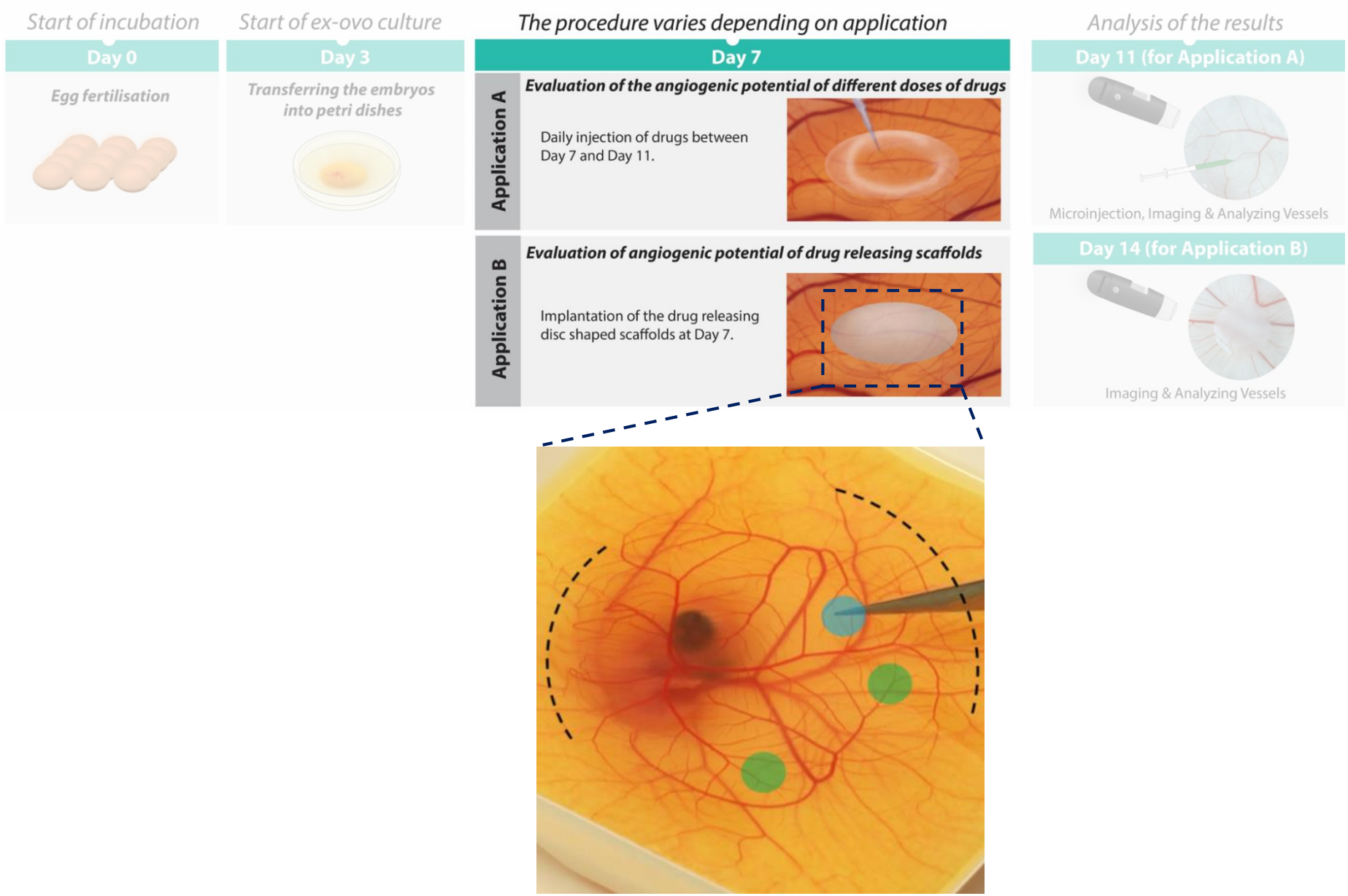


Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

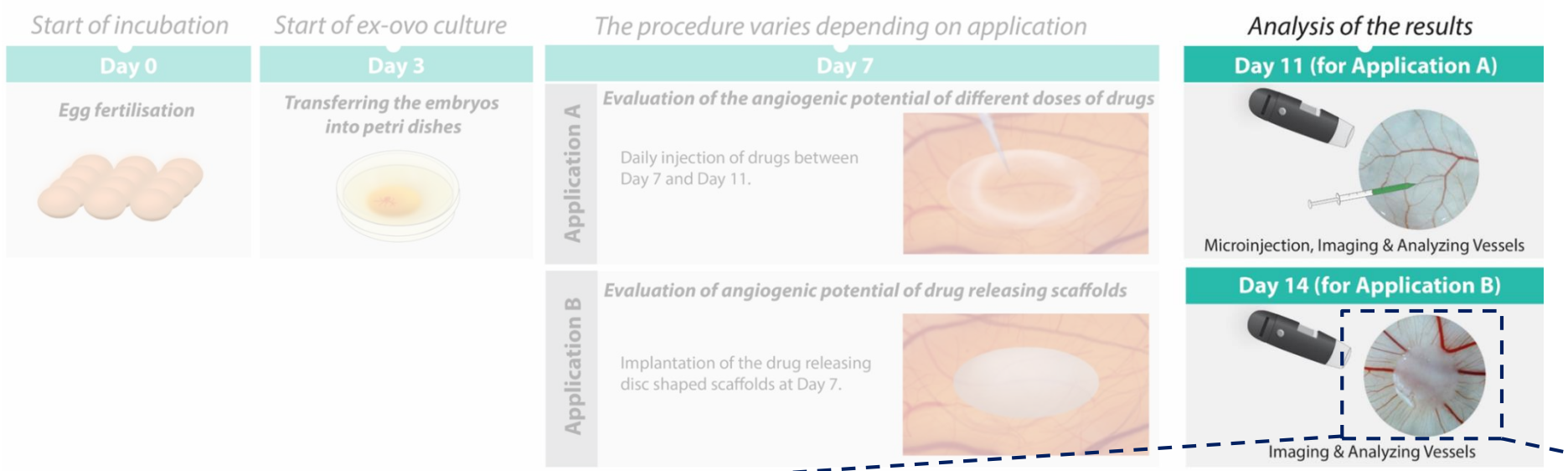


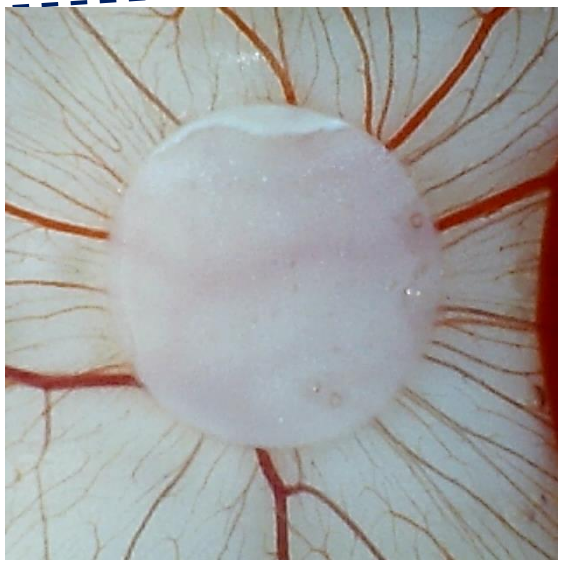


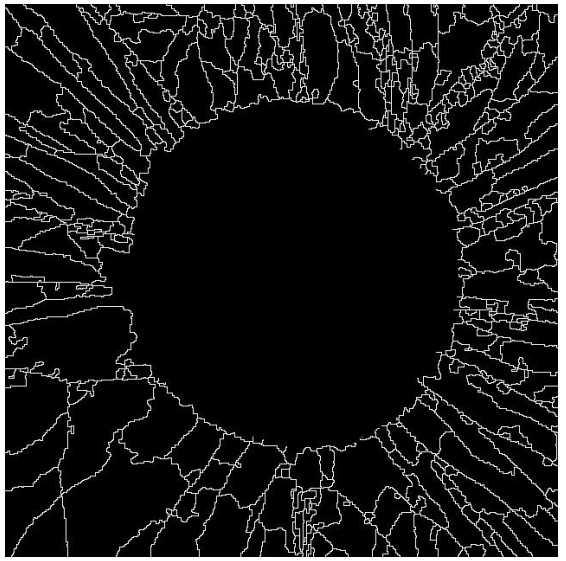
Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

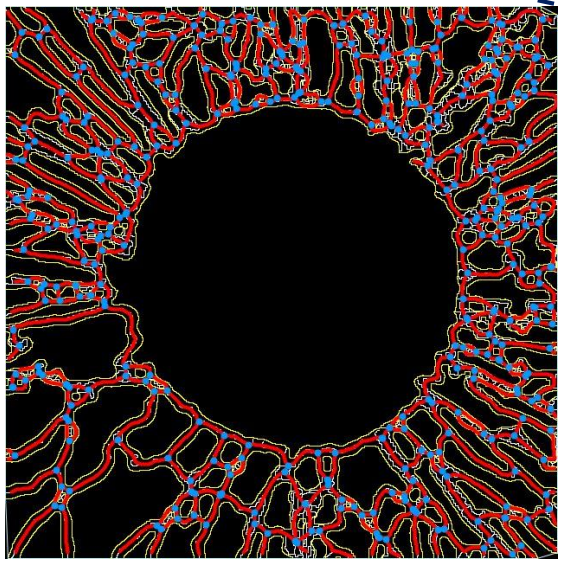


Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay





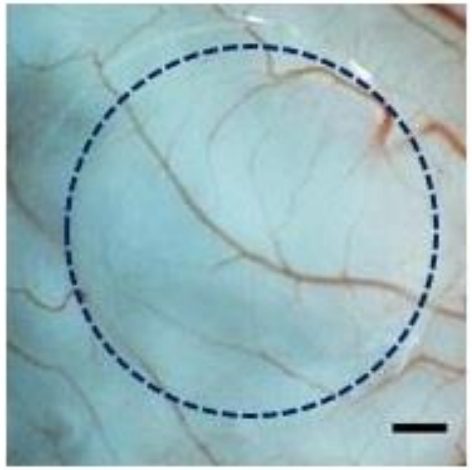


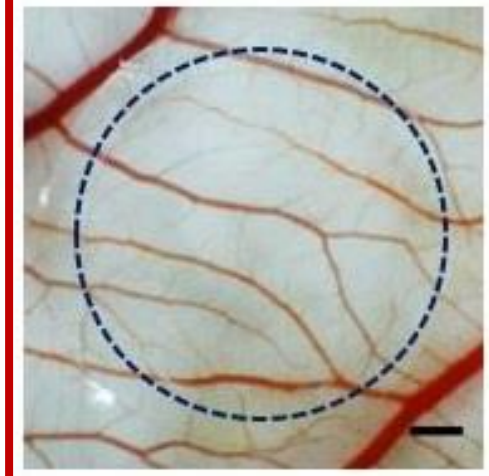


Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

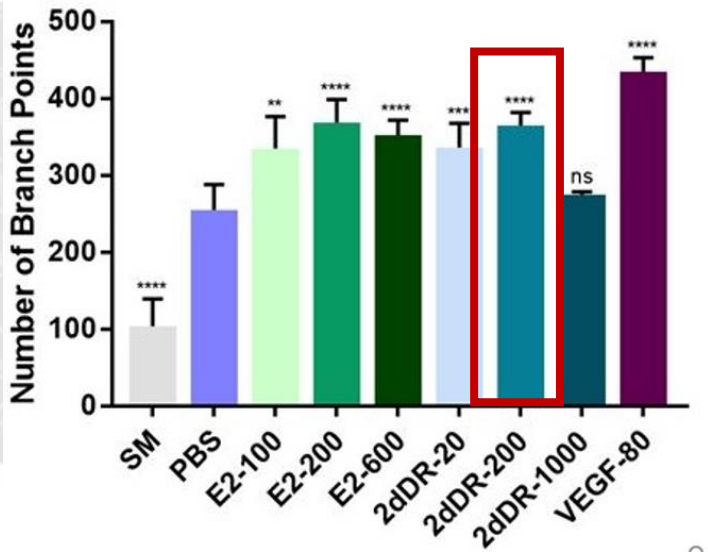
Sunitinib | **PBS** | VEGF | **2dDR** | E2

Macrovasculature





Processed images



200 µg/day/embryo

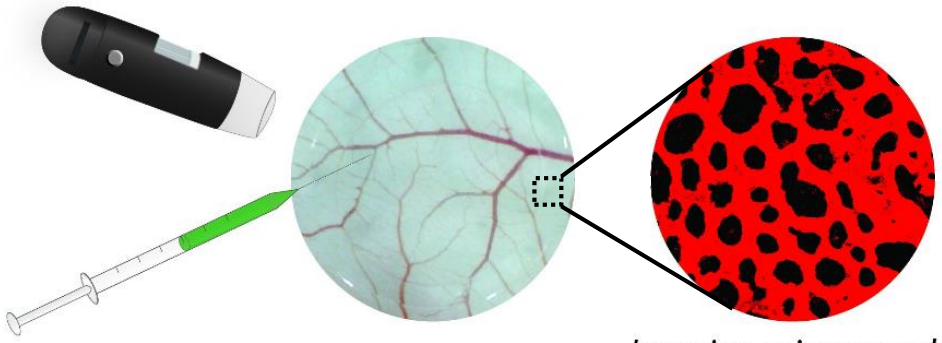
Increased the angiogenic activity **~3.6-fold** on CAM

2dDR: 2-deoxy-D-ribose;
E2: 17β-Estradiol

Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

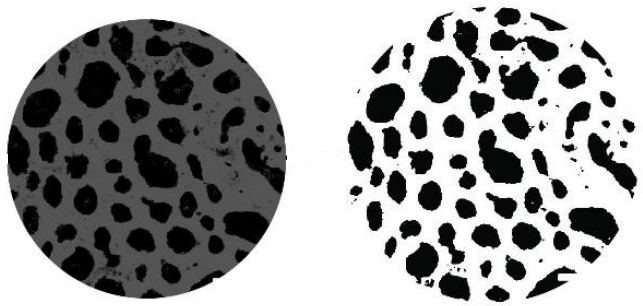
A

Microinjection



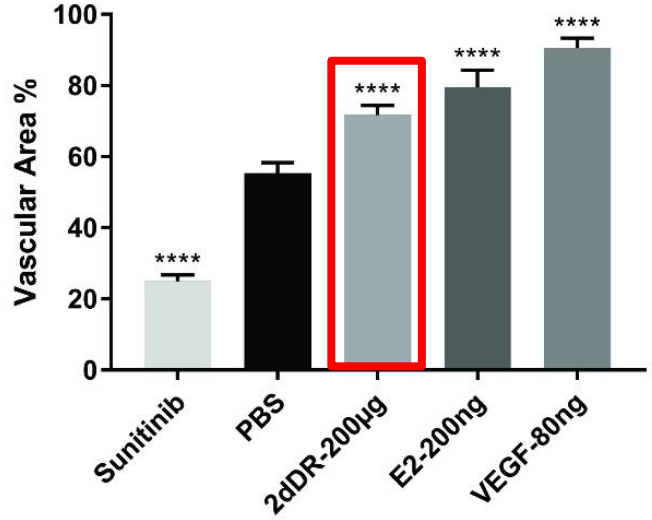
Imaging microvessels after LCA injection

Confocal imaging

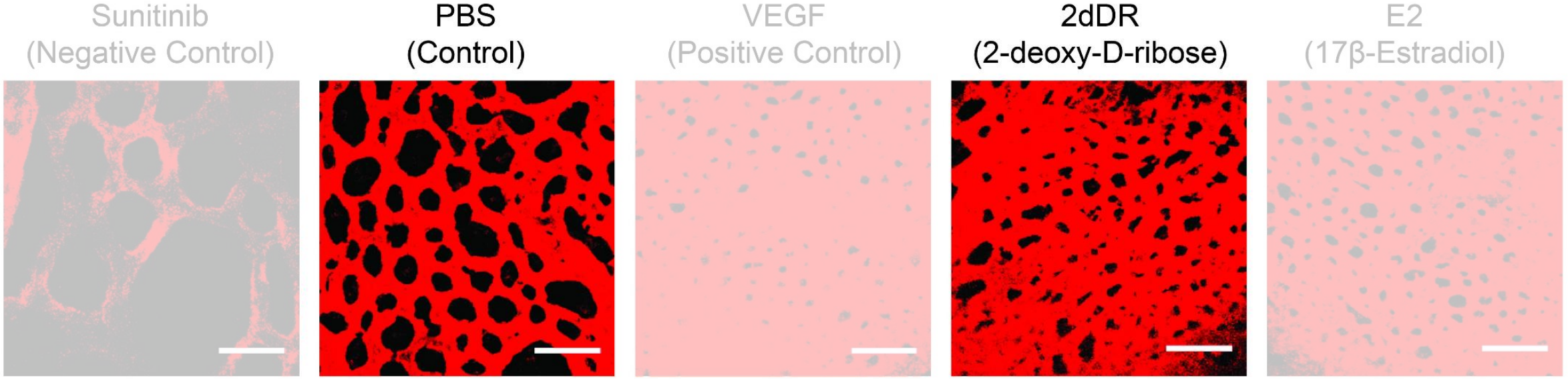


Converting the image to binary for quantification of the vascular area

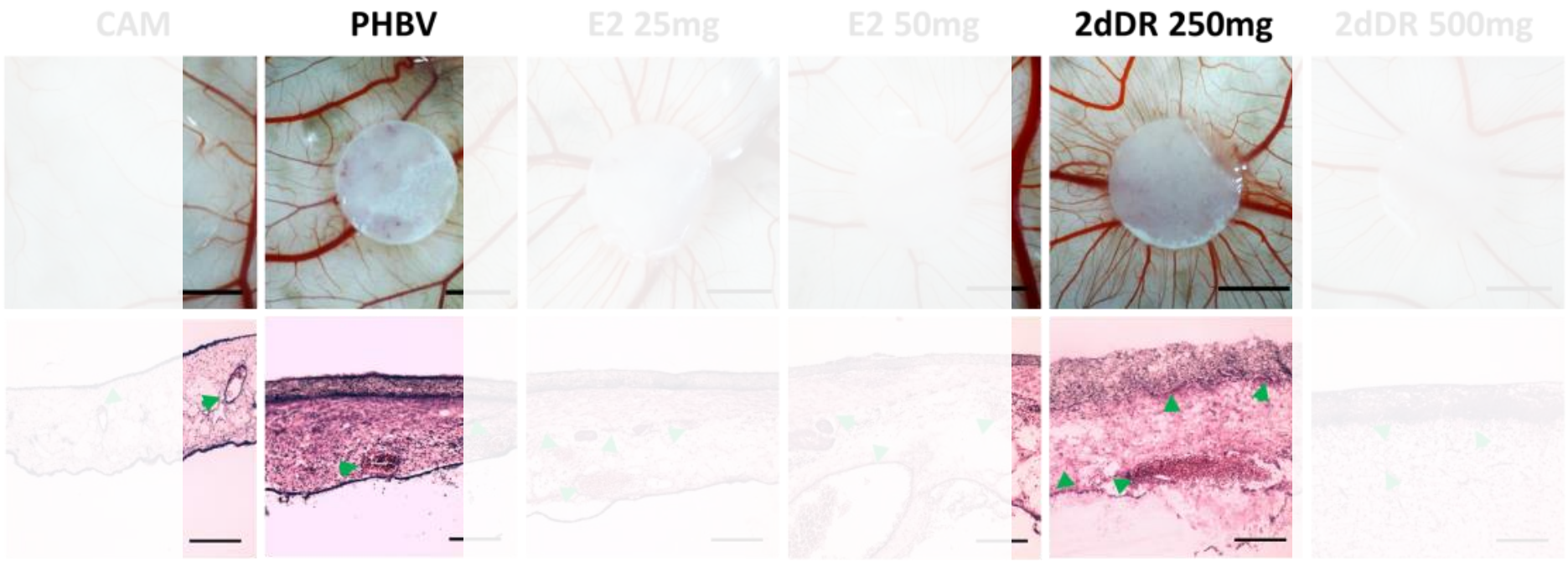
C



B

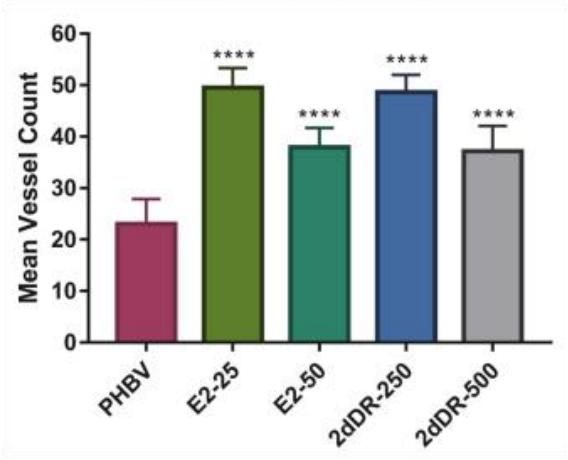


Evaluation of the angiogenic potential of 2dDR using ex-ovo CAM assay

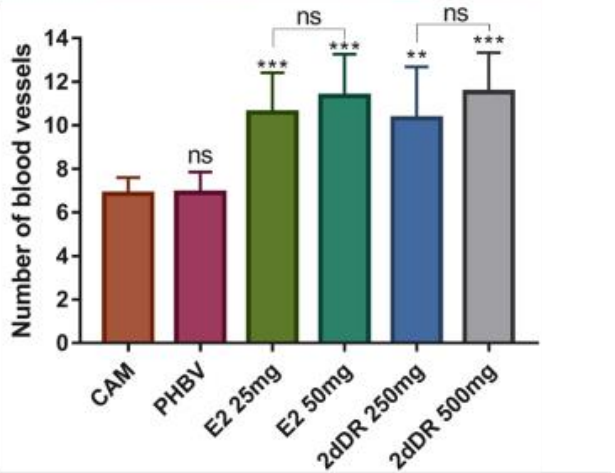


80 % of 2dDR was released within 7 days

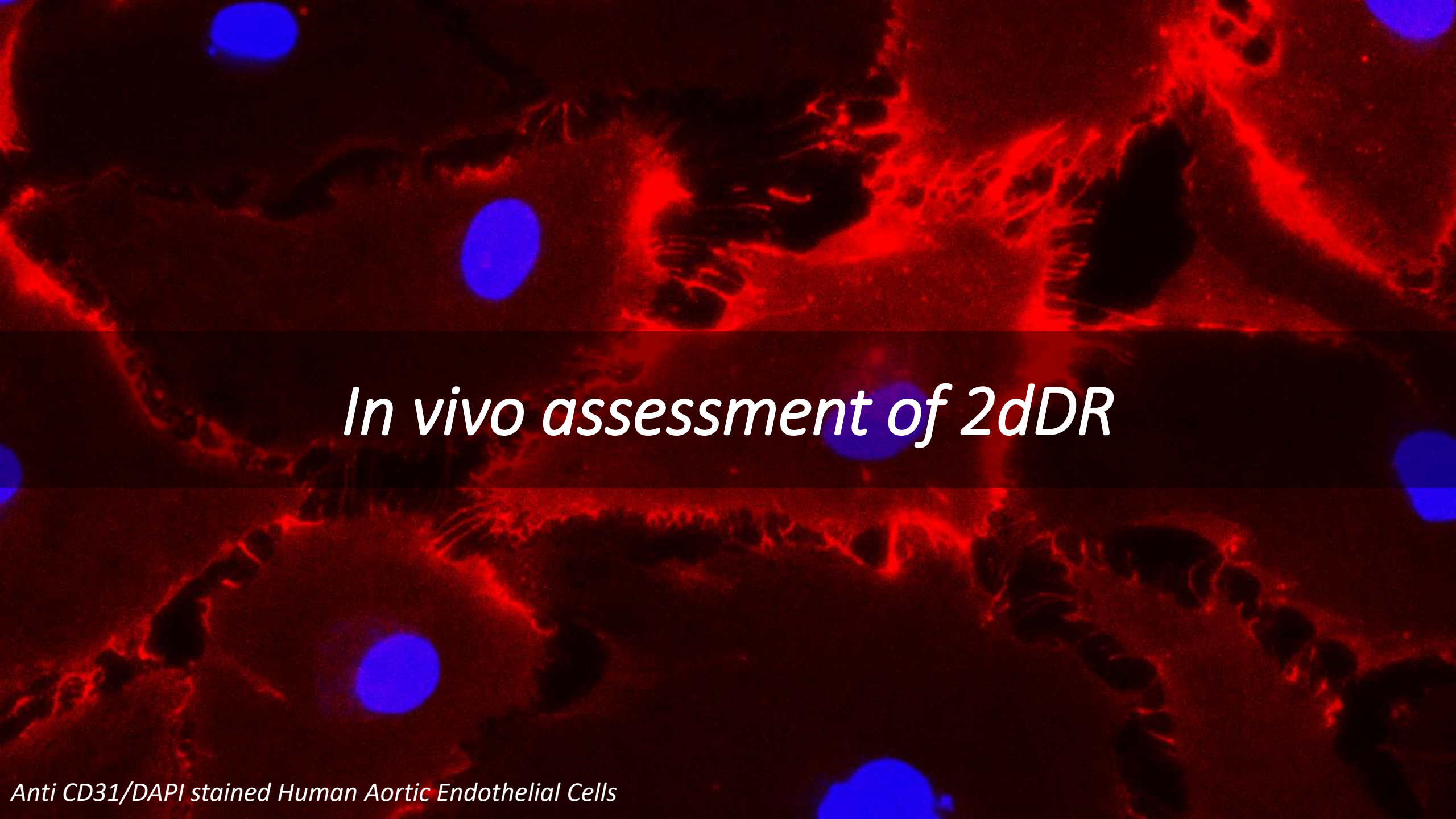
Release of 2dDR and E2 from PHBV fibers



Quantification from macro images of the scaffolds



Quantification from histology images of the scaffolds



Anti CD31/DAPI stained Human Aortic Endothelial Cells

Testing of 2dDR in diabetic rats

Total number of rats – 28

Study Groups:

1. **Sham-** (Only diabetic model with no treatment)
2. **Alginate-** (Treated with Alginate dressing only)
3. **Alginate + 2dDR (5%)-** (Treated with 5% sugar loaded Alginate dressing)
4. **Alginate + 2dDR (10%**)- (Treated with 10% sugar loaded Alginate dressing)

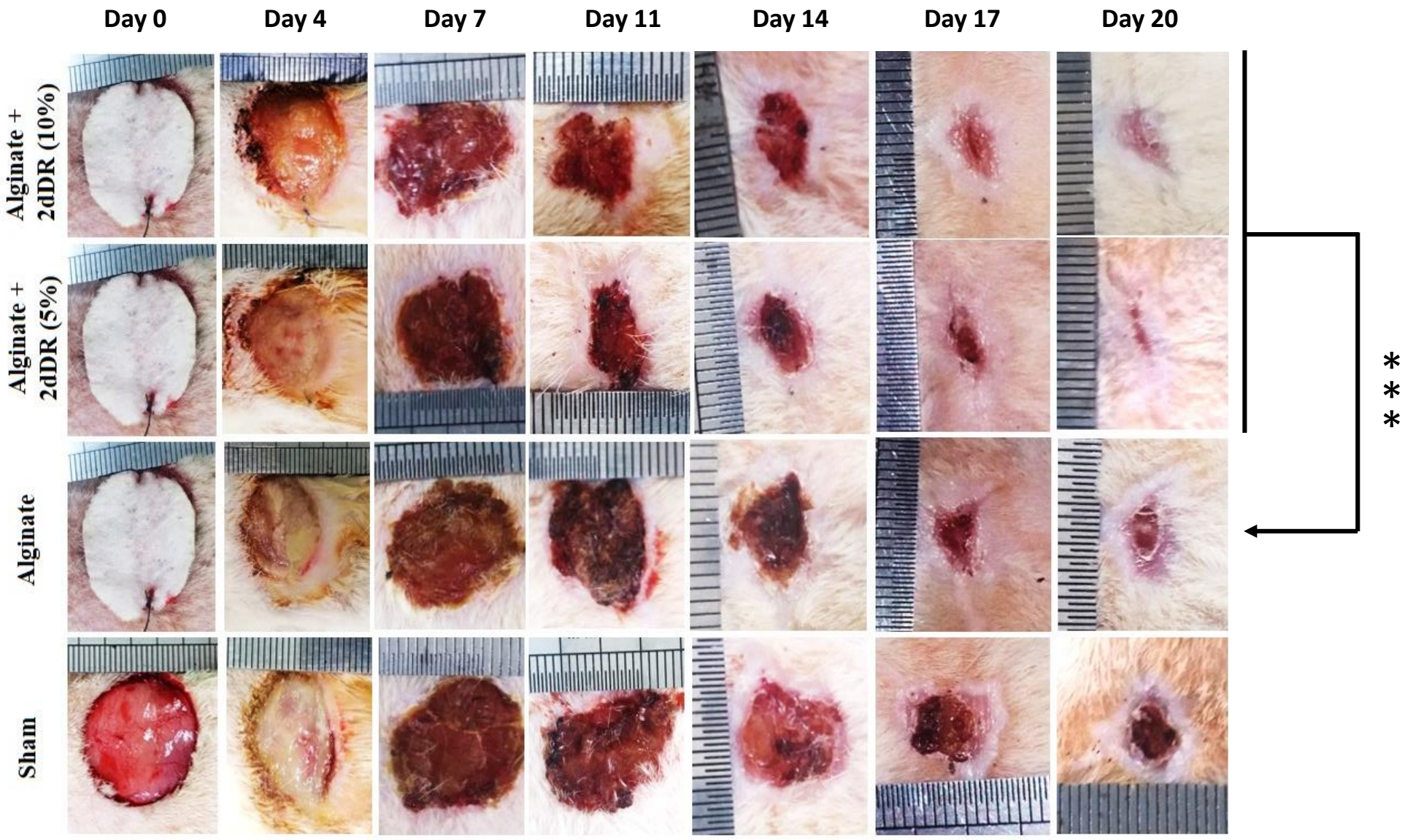
No. of rats per group= 7 rats

Wound Size= 20 mm

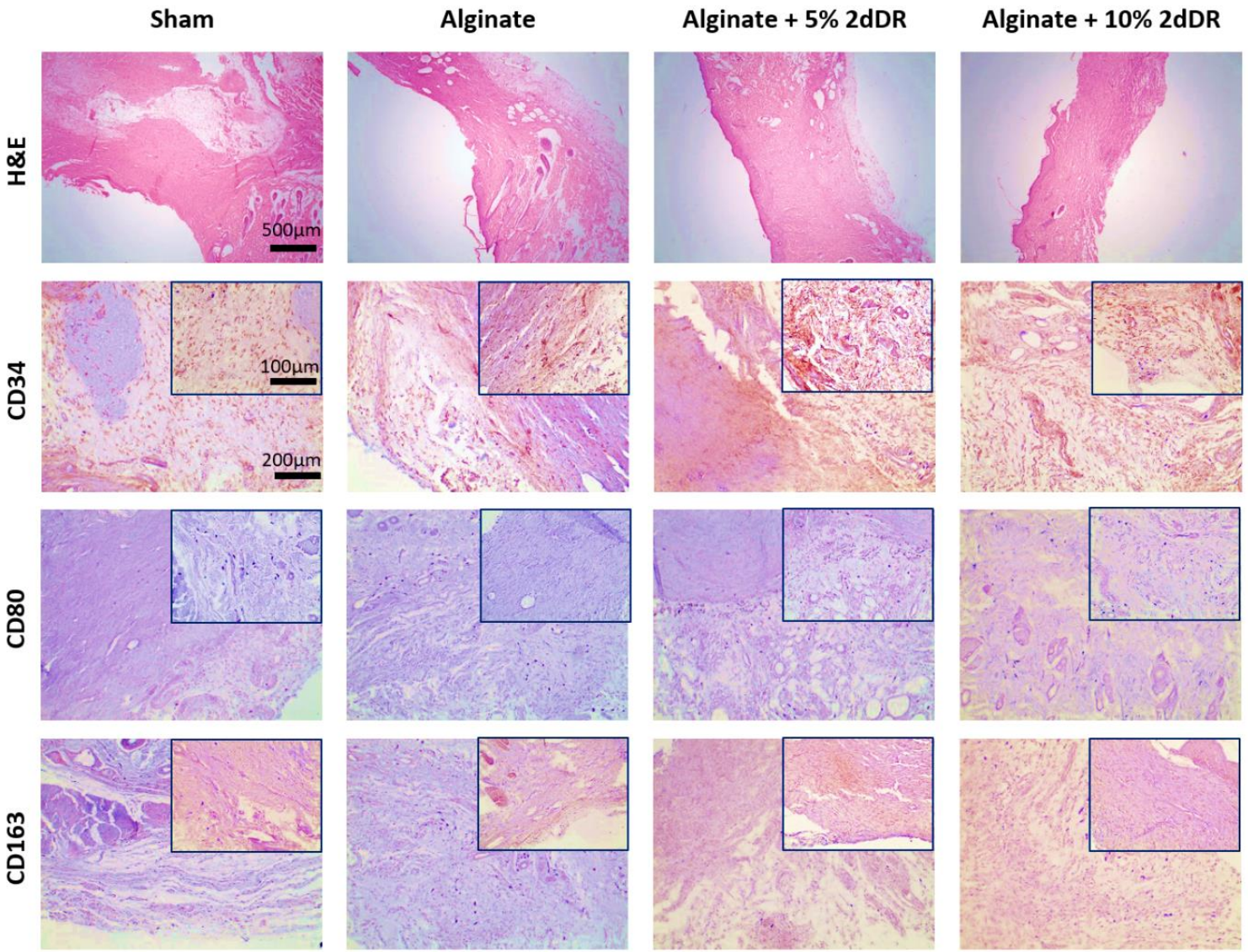
Wound Type= Full-thickness Excisional Wound

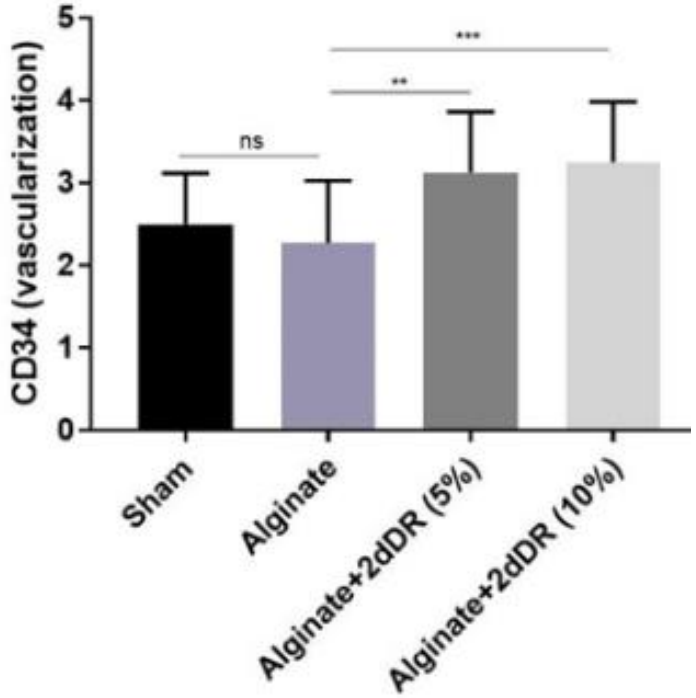
Single wound at the back of rat

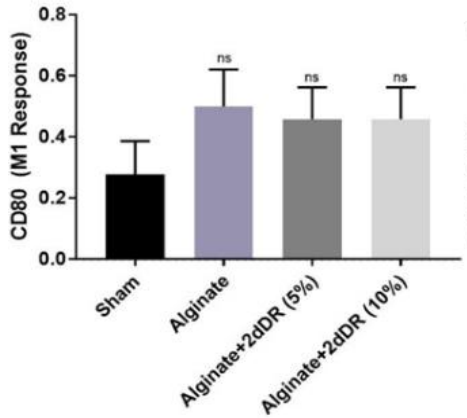
Stimulation of wound healing and angiogenesis in diabetic rats

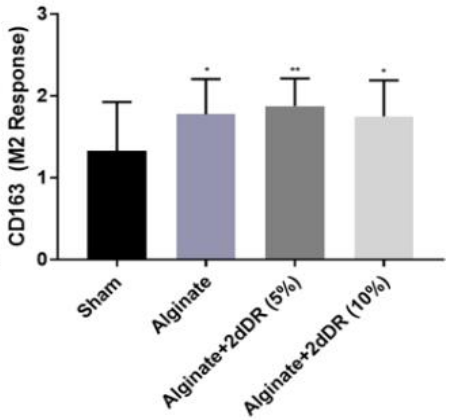


Stimulation of wound healing and angiogenesis in diabetic rats









M2 response: indicative of macrophages promoting new tissue formation
M1 response: to encapsulation and chronic inflammation thus the implant rejection

Conclusions

In vitro

- 2dDR (100 µM to 1 mM) has been found to be **~90% as effective as** VEGF
- Although the mechanism action of 2dDR is not clear , we have recently shown that 2dDR increases the VEGF production of HAECs in vitro

CAM assay

- 2dDR (200 µg/day/embryo) has been found to be **80% as potent as** VEGF in inducing angiogenesis in CAM assay.
- 2dDR can be easily loaded into and released from tissue-engineered constructs to stimulate angiogenesis in CAM assay

Diabetic rats

- The incorporation of 5% and 10% 2dDR to the alginate dressings not only stimulated wound healing and showed a full wound closure (100%), but also stimulated angiogenesis in the wound area

Conflicting literature on small sugars

Sugars	Angiogenesis Assay	Result	Effective Doses	Reference
2-deoxy-D-ribose (2dDR)	*In vitro*	Promotes proliferation, migration and tube formation of ECs	100 µM to 1 mM	[15]
		Inhibits hypoxia-induced apoptosis	10 µM	[24]
		Induces Matrigel invasion	100 µM	[25]
		Stimulates proliferation and migration of ECs	100 µM to 1 mM	[27]
		Activates NOX2 which triggers NF-κB and upregulates VEGFR2	8 µM to 1 mM	[51]
	CAM assay	Promotes angiogenesis	200 µg/day	[38]
			250 µg/1 g of polymer	[38]
			1 mg/ml	[26]
	In vivo	Promotes angiogenesis and wound healing	1 mg/ml	[26]
			5% or 10% (w/v) in the dressing	[18]
		Promotes angiogenesis	*2* nmol	[27]
2-deoxy-L-ribose (2dLR)	*In vitro*	Suppresses migration and tube formation of ECs	100 µM	[19,20]
		Inhibits VEGF production	10 µM to 100 µM	[23]
		Promotes hypoxia-induced apoptosis	30 µM to 50 µM	[24]
		Inhibits Matrigel invasion of tumours	100 µM	[25]
	CAM assay	Stimulates angiogenesis	1 mg/ml	[26]
	In vivo	Inhibits angiogenesis in a rat corneal assay	200 ng/pellet	[19]
		Promotes angiogenesis	*2* nmol	[27]
2-deoxy-D-glucose (2dDG)	*In vitro*	Inhibits the proliferation, migration and tube formation of ECs, and induces endothelial apoptosis	60 µM to 9 mM	[33]
		Inhibits proliferation of cells and reduces ATP levels	3 mM	[34]
		Inhibits the proliferation, migration and tube formation of ECs	50 µM to 1 mM	[35]
		Downregulates AKT and ERK pathways and inhibits tube formation of ECs	600 µM	[36]
	Rat aortic ring	Inhibits tube formation of ECs	50 µM to 1 mM	[35]
	In vivo	Inhibits angiogenesis	6 mM	[33]
D-Glucose (DG)	*In vitro*	Induces migration and tube formation of ECs	25 mM	[28]
		Inhibits proliferation, migration and tube formation of ECs in a dose-dependent manner	5 mM to 30 mM	[30]
		Promotes tube formation of ECs and increases COX-2 expression	25 mM to 30.5 mM	[29]
		Inhibits the tube formation of ECs in a dose-dependent manner	10 mM to 16 mM	[31]
	In vivo	Reduced angiogenesis	22 mM	[32]

Summary of presentation

- **A history of our working on 2dDR-**has spanned Pakistan, UK, USA -with funding from Turkey,Cannenta Australia, Cotton Craft Pakistan and a joint patent between the IRCB Pakistan and the University of Sheffield
- **Does it work?** In vitro , bioassay and animal studies confirm its effectiveness
- **How does it compare to VEGF in its activity and potency?** About 80-100% as effective
- **How does it work- how much do we understand it?** Evidence supporting its upregulation of VEGF


❖ Developing commercialisation route for sustained production and delivery of therapy
❖ Larger clinical study
❖ First in man safety study
❖ Obtaining regulatory approval
❖ Testing of materials in relevant animal models
❖ Designing and producing biomaterials
❖ Understanding the patient's needs

Where next?

- **We plan to move this to clinical benefit in Pakistan**-seeking funding to take it to a first in man clinical study in Pakistan

- Assembled a great team of clinicians, Industrial partner-Cotton Craft and centre to conduct pre-clinical experiments .

Joint publications

Materials Today Communications 13 (2017) 295–305

Contents lists available at ScienceDirect

Materials Today Communications

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

Deoxy-sugar releasing biodegradable hydrogels promote angiogenesis and stimulate wound healing

Muhammad Yar[a,*], Lubna Shahzadi[a], Azra Mehmood[b], Muhammad Imran Raheem[a], Sabiniano Román[c], Aqif Anwar Chaudhry[a], Ihtesham ur Rehman[a], C.W. Ian Douglas[d], Sheila MacNeil[c,*]

Regenerative Medicine

Exploration of 2-deoxy-D-ribose and 17β-Estradiol as alternatives to exogenous VEGF to promote angiogenesis in tissue-engineered constructs

Serkan Dikici[1], Naşide Mangır[1,2], Frederik Claeyssens[1], Muhammad Yar[3] & Sheila MacNeil*[,1]

Soft Tissues and Materials

JOURNAL OF biomaterials applications

Addition of 2-deoxy-D-ribose to clinically used alginate dressings stimulates angiogenesis and accelerates wound healing in diabetic rats

Maryam Azam[1], Serkan Dikici[2], Sabiniano Roman[2], Azra Mehmood[3], Aqif A Chaudhry[1], Ihtesham U Rehman[4], Sheila MacNeil[2] and Muhammad Yar[1]

Journal of Biomaterials Applications
0(0) 1–13

DOI: 10.1177/0885328219859991
journals.sagepub.com/home/jba
SAGE

frontiers

Assessment of the angiogenic potential of 2-deoxy-D-ribose using a novel in vitro 3D dynamic model in comparison with established in vitro assays

Serkan Dikici[1, 2], Betül Aldemir Dikici[1, 2, 3], Shirin I. Bhaloo[1], Mercedes Balcells[1, 4], Elazer R. Edelman[1, 5], Sheila MacNeil[2], Gwendolen C. Reilly[2, 3], Colin Sherborne[2], Frederik Claeyssens[2*]

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Cannenta ADVANCED WOUND CARE

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