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

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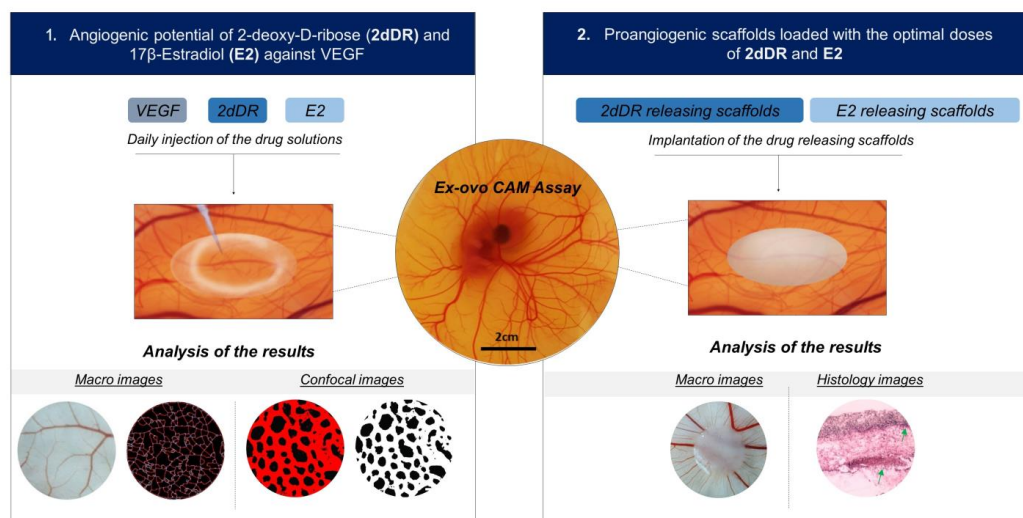
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Abstract:

Aim: In this study, we explored the angiogenic potential and proangiogenic concentration ranges of 2-deoxy-D-ribose (2dDR) and 17 β -Estradiol (E2) in comparison with vascular endothelial growth factor (VEGF). 2dDR and E2 were then loaded into tissue engineering (TE) scaffolds to investigate their proangiogenic potential when released from fibres. **Materials and Methods:** *Ex-ovo* chick chorioallantoic membrane (CAM) assay was used to evaluate angiogenic activity of 2dDR and E2. Both factors were then introduced into scaffolds via electrospinning to assess their angiogenic potential when released from fibres. **Results:** Both factors were approximately 80% as potent as (VEGF) and showed a dose-dependent angiogenic response. The sustained release of both agents from the scaffolds stimulated neovascularisation over 7 days in the CAM assay. **Conclusion:** We conclude that both 2dDR and E2 provide attractive alternatives to VEGF for the functionalisation of TE scaffolds to promote angiogenesis *in vivo*.

Graphical abstract:



Keywords: angiogenesis; chick chorioallantoic membrane assay; 2-deoxy-D-ribose; estradiol; VEGF

1. INTRODUCTION

Over the last 30 years there have been significant advances in the production of tissue engineered materials suitable for use in the clinic. However, one of the key challenges is to ensure rapid neovascularisation into these constructs in order for them to survive post transplantation [1]. While relatively thin simple tissue engineered constructs can survive on well-vascularised wound beds, thicker constructs (>200 μm) usually fail to engraft due to lack of oxygen and nutrients *in vivo* [2,3]. Both prevascularisation and scaffold functionalisation strategies with the use of angiogenic factors are viewed as promising approaches to accelerate vascular ingrowth into tissue engineering (TE) constructs to circumvent slow vascularisation after implantation [1,4].

Although there are some well-known growth factors such as transforming growth factor-beta (TGF- β), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) which have the potential to promote neovascularisation [5], vascular endothelial growth factor (VEGF) is recognised to be the most well-studied angiogenic factor due to occupying a key role in the angiogenic cascade. VEGF has been proven to have important roles in different steps of the angiogenic process *in vivo*: vasodilation and permeability, destabilization of vessels and degradation of extracellular matrix (ECM), proliferation and migration of endothelial cells, and lumen formation and vessel stabilisation [6,7].

VEGF acts as part of a well-regulated process, and its actions are highly dose-dependent. It is largely bound to glycosaminoglycans *in vivo* and released in response to need. Its angiogenic potential has been assessed in many *in vitro* studies such as cell migration assays using matrigel [8], collagen gels [9] and transwell migration assays [10]. It has also been evaluated in the CAM assay [11–14] as well as *in vivo* studies. However, a range of studies show that VEGF addition can lead to excessively leaky [15], permeable [16] and haemorrhagic [17] vessels such as those that are found in tumorigenesis [18]. Controlled and slow release of VEGF may help to regulate the delivery rate of VEGF and circumvent these problems by creating mature, more durable and stable vessels [19,20]. One promising approach is to use the glycosaminoglycan heparin, which is found on the cell surface and in ECM [21], to bind VEGF. Heparin found in ECM plays a role in storage and prolonging the release of heparin binding growth factors such as FGF and VEGF. It also regulates their stability and biological activity as well as long-term stimulation of endothelial cells [22–24]. Our group has explored the approach of using heparin bound to biomaterials to deliver VEGF using a layer-by-layer method for coating scaffolds with heparin and then binding VEGF [25] and we have also reported on chitosan-based hydrogels for binding heparin [26,27]. However, binding VEGF with heparin is a long process and requires multistep actions to introduce VEGF with the TE scaffolds.

As an alternative to VEGF, 17 β -Estradiol (E2) has been shown to promote endothelial cell migration and proliferation *in vitro* [28,29] and to stimulate new blood vessel formation both *in vitro* and *in vivo* [30]. E2 has an important role in neovascularisation during the menstrual cycle [31,32]. It is used clinically in the treatment of osteoporosis and heart disease [33]. Moreover, blocking the E2 receptor with adjuvants such as tamoxifen for estrogen receptor positive tumours, in which high estrogen helps the cancer cells grow and spread, is an effective method to reduce tumour vasculature. This therapy has been in clinics for many years especially for treatment of breast cancer [34,35]. Recently, our group confirmed that poly-L-lactic acid (PLLA) scaffolds loaded with E2 were highly angiogenic using the CAM assay [36].

Thymidine phosphorylase (dThdPase) has an amino acid sequence identical to platelet-derived endothelial cell growth factor (PD-ECGF) and is an enzyme that catalyses the conversion of thymidine to thymine [37,38]. Activation of dThdPase is known to be angiogenic [39–41] although its molecular mechanism is still unclear. 2-deoxy-D-ribose (2dDR), one of the degradation products of thymidine, was reported to have a chemotactic activity and angiogenic activity *in vivo* [42,43]. However, no other similar molecules including thymidine, thymine, 2-deoxy-L-ribose, 2-deoxy-D-ribose-1-phosphate were found to be angiogenic when compared to 2dDR [43,44]. Similarly, our group previously found that 2dDR, released from a hydrogel, is not only angiogenic but also stimulates wound healing in a rat skin wound model [45]. However, as only a single dose of 2dDR was used in that study, dose-dependent response to 2dDR remained still unclear.

In summary, although VEGF is a highly effective stimulator of angiogenesis, it has also been demonstrated to be unstable, expensive and possibly unsafe when given in a non-regulated manner. Thus, there is need to explore alternative strategies for positively influencing the angiogenic cascade due to the potential drawbacks of using exogenous VEGF. E2 has proven as safe to be used clinically. To date, the angiogenic response to different doses of E2 has been studied by several groups [46–48]. On the other hand, although potential angiogenic effect of 2dDR has been previously reported, there have not been any studies so far on the concentration range of 2dDR which is proangiogenic.

Accordingly, our aim in this study is to establish useful proangiogenic concentration ranges for both 2dDR and E2 and to compare their angiogenic activities with VEGF to progress our understanding of the potential value of E2 and 2dDR to the world of proangiogenic biomaterials and to the problem of improving rapid neovascularisation in tissue engineered constructs. In order to satisfy this aim, we explored the angiogenic potential of different concentrations of 2dDR and E2 in order to determine the effective dose ranges of both agents which were found approximately 80% as potent as VEGF for promoting angiogenesis when applied directly on CAM. Following the determination of the effective doses of the 2dDR and E2, both drugs were loaded into a simple electrospun scaffold of Poly3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) fibres from which they were readily released. PHBV was selected due to its biodegradability and good biocompatibility which can be linked with its degradation product is 3-hydroxybutyrate that is also a natural product produced in human body [49]. The release of both agents from fibres also showed an increased angiogenic activity in CAM assay and stimulated neovascularisation *in vivo*.

Therefore, for the first time in this study, we defined effective pro-angiogenic concentration ranges of 2dDR and compared it with E2 and VEGF in CAM assay in which one can demonstrate significant increases in blood vessel formation within seven days, which is a time period very relevant to driving angiogenesis non-healing chronic wounds *in vivo* and to stimulating the formation of new blood vessels after engraftment of TE constructs.

2. MATERIALS AND METHODS

2.1. *Ex-ovo* CAM assay

2.1.1. Incubation of eggs

All CAM experiments were carried out according to the Home Office, UK guidelines. Schematic demonstration of the *ex-ovo* CAM assay is given in Figure 1. Fertilised chicken eggs (*Gallus domesticus*) were purchased from Henry Stewart & Co. (MedEggs, Norwich, UK). The eggs were carefully wiped with 20% industrial methylated spirit solution using hand paper towels to remove dirt and feathers from the shell. The eggs were then incubated at 37.5 °C until embryonic development day (EDD) 3, lying horizontally in a humidified egg incubator (RCOM King SURO, P&T Poultry, Powys, Wales).

2.1.2. Transferring the embryos into petri dishes

On EDD 3, the upper surface of the eggs was marked with a felt pen. The eggs were held horizontally (with the marked surface on top) and cracked on the edge of a 1000 ml glass beaker and kept close to the bottom surface of the petri dishes. The embryos were then transferred gently into sterile petri dishes and kept in a humidified incubator (Binder, Tuttlingen, Germany) at 38°C.

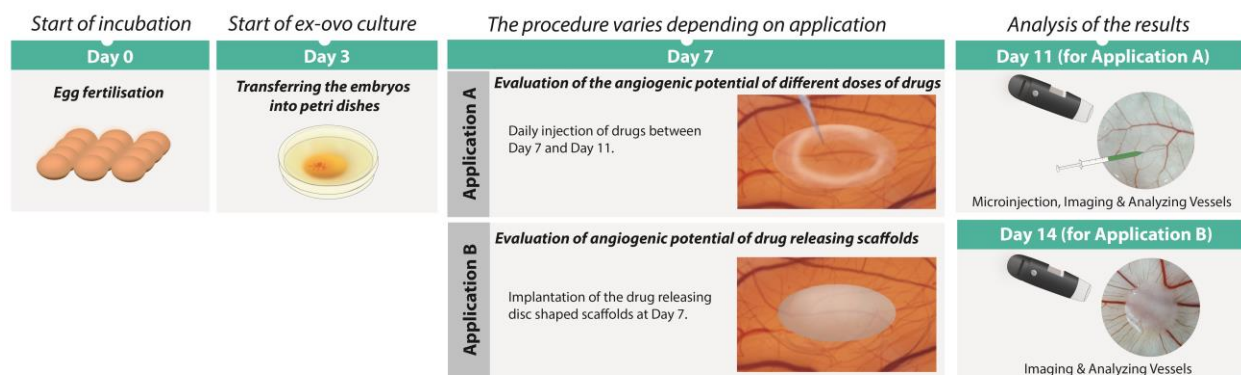


Figure 1. Schematic illustration of the steps of the direct application of the substances and implantation of the substance releasing scaffolds on CAM. This figure shows the basic methodology of *ex-ovo* CAM assay and quantification of the macro and micro images.

2.2. Determination of the optimum concentration of E2 and 2dDR on angiogenesis using the CAM assay

2.2.1. Preparation of drug solutions

Three concentrations of E2 and 2dDR were screened in these experiments. E2 was dissolved in methanol then working solutions were prepared with phosphate buffered saline (PBS) so as to be (a) 100 ng/day (E2-100), (b) 200 ng/day (E2-200), and (c) 600 ng/day (E2-600) concentrations. 2dDR solutions were prepared by dissolving in PBS so as the final concentrations to be (a) 20 µg/day (2dDR-20), (b) 200 µg/day (2dDR-200), and (c) 1000 µg/day (2dDR-1000). VEGF was used as a positive control at a concentration of 80 ng/day (VEGF-80) whereas Sunitinib, an inhibitor of multiple receptor tyrosine kinases, was used as a

negative control at a 2 µg/day concentration. Working solutions of all substances were prepared freshly at the beginning of each experiment. All chemicals were purchased from Sigma Aldrich unless indicated otherwise.

2.2.2. Application of drugs onto the CAM

Plastic rings (~6.5 mm in diameter), as a reservoir for the drugs and a marker for the implantation area, were placed on the CAM. The substances were applied as 20 µl volume onto the CAM twice a day for 3 days starting from EDD 7. On EDD 11, images of the CAM area circumscribed by the plastic rings were acquired using a digital microscope and embryos were sacrificed immediately after image acquisition.

2.2.3. Quantification of angiogenesis

Digital images were used for quantification of the results. Multiple image processing steps were applied following previously described protocols [6,7,50]. Firstly, the internal area of the ring was cropped, and the raw image was split to its three main colour channels (red, green and blue (RGB) channels) using Adobe Photoshop CS6 (ADOBE Systems Inc., San Jose, California, USA). Only the green channel was exported as an image file and then imported to ImageJ (Wayne Rasband, National Institutes of Health, USA) for further analysis including unsharp mask filtering, enhancing the local contrast, noise removal, converting the image to binary and segmentation. The green channel was selected because it gave the most accurate and detailed results for blood vessels when converted to binary [51]. Finally, the number of branch points was quantified using quantification software (AngioTool, National Cancer Institute, USA) and average blood vessel lengths were calculated by using binary image histograms with known pixel/mm ratios in ImageJ (Wayne Rasband, National Institutes of Health, USA).

2.3. Evaluation of effect of E2 and 2dDR on microvasculature

For these experiments only, one concentration of E2 (E2-200) and 2dDR (2dDR-200) were used as these concentrations were the most effective ones in terms of stimulating angiogenesis in CAM assay. The drugs were applied following the steps in section 2.2.2.

2.3.1. Microinjection

At EDD 11, a 20% solution of rhodamine labelled lens culinaris agglutinin (LCA) (Vector Laboratories, Peterborough, UK) was injected into the circulation of CAM using 30 G needles under a dissection microscope (Wild Heerbrugg, Heerbrugg, Switzerland) to visualize the microvasculature. After 1 min of incubation, embryos were sacrificed and areas on CAM circumscribed by the plastic ring were removed and fixed in 3.7% formaldehyde solution. Fixed CAM samples were then imaged under a confocal microscope (Zeiss LSM 510 Meta, Jena, Germany) for investigating the effect of substances on the microvascular structure of the CAMs.

2.3.2. Quantification of the vascular area

The percentage vascular areas (VA%) of the microvasculature of CAMs were quantified using confocal images of LCA injected CAM samples as shown in Figure 3. The images were then imported to ImageJ and

converted to binary images after filtering and smoothing processes prior to quantification. VA% was calculated using the histogram list of black and white areas in the image.

2.4. Construction of E2 and 2dDR releasing PHBV scaffolds

2.4.1. Electrospinning E2 and 2dDR loaded PHBV scaffolds

Preparation of the solutions

10% (w/w) PHBV solution was prepared prior to electrospinning. 1 g of PHBV granules (Goodfellow, London, UK) were dissolved in 1 g of methanol (Fisher Scientific, Massachusetts, USA) and 8 g of DCM (Fisher Scientific, Massachusetts, USA) in a fume hood. Four 10% PHBV solutions were prepared prior to addition of the drugs. Finally, 25 mg E2, 50 mg of E2, 250 mg of 2dDR and 500 mg of 2dDR were then added to each solution per 1 g of PHBV. The solutions were mixed using magnetic stirrer overnight.

Electrospinning

Solutions (~10 ml) were loaded into 10 ml syringes fitted with 0.6 mm inner diameter syringe tips. Syringes were then placed in a syringe pump (GenieTMPlus, KentScientific, Connecticut, USA). Aluminium foil was used as the collector and placed at a distance of 17 cm from the needle tips. The pump was set to 40 μ l/min, and 17 kV voltage was applied both to the collector and the tips. Electrospinning was done at room temperature until all the polymer solution was used.

2.4.2. Characterization of the scaffolds

Scanning electron microscopy (SEM)

The surface morphology of E2 and 2dDR releasing scaffolds were observed under SEM (Philips/FEI XL-20 SEM; Cambridge, UK). The samples were coated with gold using a gold sputter (Edwards sputter coater S150B, Crawley, England) prior to imaging. Fiber diameter and pore sizes were measured using ImageJ.

E2 and 2dDR release from the scaffolds

Scaffolds were cut into pieces so to fit into a 6-well plate, weighed and submerged in 4 ml of PBS. The accumulative E2 and 2dDR concentrations released from each group (25 mg E2, 50 mg E2, 250 mg 2dDR, 500 mg 2dDR) were measured fluorometrically using a UV-VIS spectrophotometer (Thermo Fischer Evolution 220, Massachusetts, USA) at 238 nm for 2dDR and 220 nm for E2. Absorbance values were converted into concentrations using a standard curve of known concentrations of E2 and 2dDR.

Effect of the drugs on mechanical properties of the scaffolds

Biomechanical testing samples were prepared by cutting 20 mm x 10 mm pieces from dry scaffolds. The clamps of the device were positioned 10 mm away from each other, and the width and thickness of each scaffold were measured. Test samples were clamped with two grips in a tensiometer (BOSE Electroforce Test Instruments, Minnesota, USA). Tensile tests were performed on each sample at a rate of 0.1 mm/s until the samples failed (n=4). The raw data of these tests were used for drawing stress-strain and load-

displacement graphs. Ultimate tensile strength (UTS) was calculated from stress (σ) and strain (ϵ) curves of each sample, while stiffness was calculated from load (F) and displacement (ΔL) data.

Wettability tests of drug releasing electrospun scaffolds were also undertaken using a drop shape analyser (Krüss DSA100, Germany) under ambient laboratory conditions in order to see the effect of E2 and 2dDR on wettability of the scaffolds. In brief, a 5 μ l water droplet was dropped onto the scaffold surface, and the retention times of the droplet on scaffolds before complete absorption were calculated from recorded movies of the tests. At least nine measurements (three drops on three different substrates) were taken for measuring the water retention time on each sample.

2.5. Evaluation of angiogenic potential of the E2 and 2dDR releasing electrospun PHBV scaffolds

2.5.1. Implantation of the E2 and 2dDR releasing scaffolds on CAM

Scaffolds were cut into 5.5 mm diameter circles using a laser cutting machine (Epilog Laser Cutter, Clevedon, UK) and sterilised under UV light for 1 hour prior to implantation. Two circular scaffolds were placed on CAM at EDD 7 and embryos were cultured for further 7 days.

2.5.2. Quantification of the angiogenic activity of the drug releasing scaffolds

Images of the scaffolds implanted on CAM were acquired using a digital microscope at EDD 14. Embryos were then sacrificed and scaffolds were cut together with a rim of surrounding CAM tissue and fixed in 3.7% formaldehyde solution. Angiogenesis was quantified by counting all blood vessels growing towards the scaffolds in a spoke wheel pattern, as described previously [36].

2.5.3. Histological evaluation of the E2 and 2dDR releasing scaffolds on CAM

Haematoxylin and Eosin (H&E) staining was performed on cell impregnated scaffolds by modifying a standard protocol [52]. Briefly, fixed samples were embedded in optimal cutting temperature freezing medium and frozen in liquid nitrogen for 3 minutes. Sections were cut 8-10 μ m thick using a cryostat (Leica Biosystems Nussloch, Germany) at -20°C. Sections were then stained with haematoxylin for 90 seconds and eosin for 5 minutes. Finally, H&E images were acquired under a light microscope (Motic DM-B1, Xiamen, China). The total number of blood vessels adjacent to the scaffolds were quantified by counting blood vessels in H&E sections [53]. Briefly, all discernible blood vessels adjacent to the scaffolds were counted by two independent researchers using two independent microscopes at 10 \times magnification. Three independent CAM experiments were conducted, and in each independent experiment, six embryos were used for each group. For histological analysis of a single group, three embryos were randomly selected out of six embryos from each independent experiment. Six sections were taken on a slide from each of the nine samples, and each slide was investigated under a microscope making a total of 54 counts per group for quantification of the results.

2.6. Statistics

Statistical analyses were performed using unpaired student's t-test. P values <0.05 were considered as statistically significant, and the degree of significance was indicated with number of stars, **** P ≤ 0.0001, *** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05, ns P ≥ 0.05.

3. RESULTS

3.1. Assessment of angiogenic activity of E2 and 2dDR on CAM

Quantification of the macroimages of CAMs showed that E2-100, E2-200 and E2-600 groups increased the number of branch points 1.3-fold, 1.5-fold and 1.4-fold respectively. All concentrations increased the average vessel length approximately 1.2-fold compared to controls over 4 days. In the same way, 2dDR-20 and 2dDR-200 increased the number of branch points by 1.3 times and 1.4 times, respectively. Both concentrations increased the average vessel length approximately 1.2 times while there was no significant difference for the 2dDR-1000 group compared to control scaffolds. VEGF increased the number of branch points by 1.4-fold and the average vessel length by 1.7-fold. Quantification of the branch points, average vessel lengths and the macroimages of the CAMs with the most effective concentrations of E2 (E2-200) and 2dDR (2dDR-200) are shown in Figure 2.

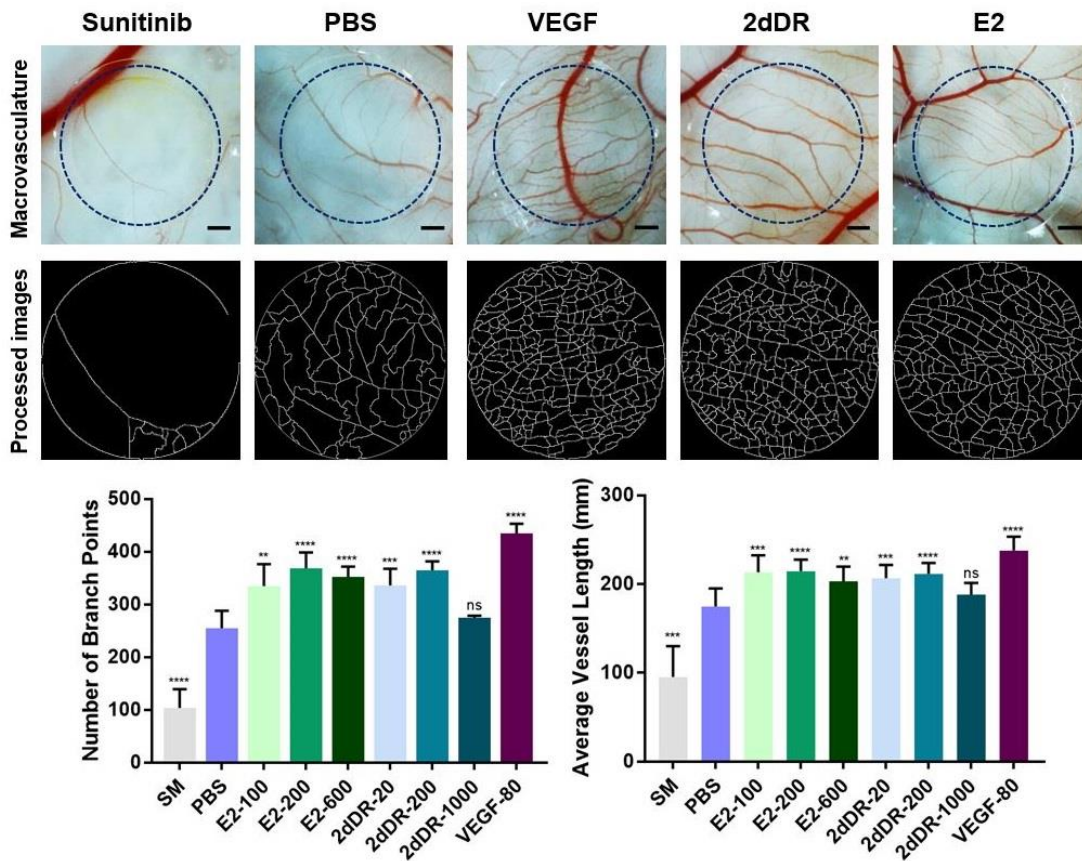


Figure 2. Evaluation of different concentrations of E2 and 2dDR to stimulate new blood formation in a chorioallantoic membrane assay. Three concentrations of both drugs were compared against a negative

(Sunitinib), PBS and positive (VEGF) control in these experiments. A normal angiogenic response can be seen in the PBS group. A significant increase in the average length of blood vessels and number of branch points compared with PBS can be observed for both E2 and 2dDR groups (upper row). Representative images are given for E2 (200 ng/day) and 2dDR (200 µg/day). Processed images used for quantification of results are given for each group (middle row). **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, ns $p \geq 0.05$, $n = 9 \pm$ standard deviation. The number of branch points and average macrovessel lengths seen in response to different concentrations of E2 and 2dDR were calculated and compared with PBS controls over 4 days (lower row). Values represent mean \pm standard deviation. Scale bars represent 1 mm.

2dDR: 2-Deoxy-ribose; E2: 17 β -Estradiol; ns: Non significant; PBS: Phosphate-buffered saline

3.2. Effect of E2 and 2dDR on the microstructure of CAM

Microvascular evaluation of the CAM samples showed that VA% was increased from $55.3 \pm 3\%$ to $79.5 \pm 5\%$ and $71.7 \pm 3\%$ for E2 and 2dDR applied groups respectively compared to controls over 4 days. VEGF and Sunitinib were used as positive and negative controls. Although the VA% was significantly higher in VEGF groups when compared with 2dDR and E2 applied CAMs, VEGF also resulted in smaller lacunae formation. Sunitinib, as an inhibitor of angiogenesis, led to much larger lacunae (Figure 3).

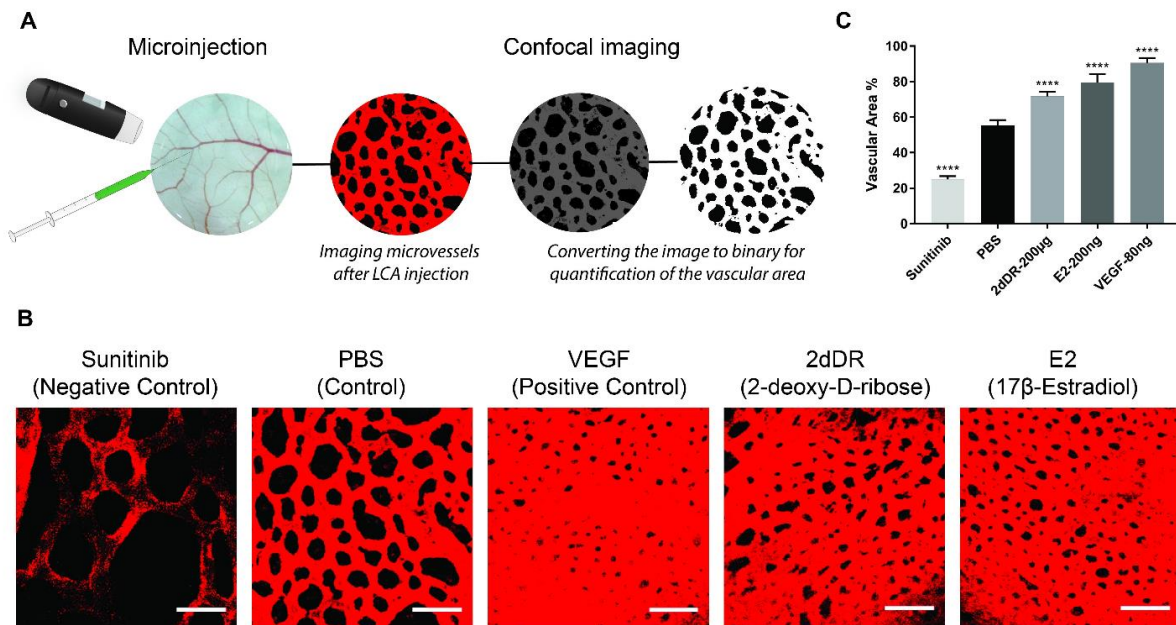


Figure 3. Demonstration of the effect of Estradiol (E2) and 2-deoxy-D-ribose (2dDR) on microvasculature compared with the application of phosphate-buffered saline (control). Rhodamine-labelled LCA was injected into the circulation of the chorioallantoic membrane to visualize microvasculature. The vascular areas were labelled with red colour which indicates LCA-positive areas (A). Similar to VEGF group, an endothelial cell hypertrophy together with smaller lacunae compared with PBS can be observed for both E2 and 2dDR groups (B). Percentage area covered by endothelial cells (microvasculature) were calculated for each group and compared (C). The 2dDR was applied as 200 µg/day and E2 as 200 ng/day. **** $p \leq 0.0001$, *** $p \leq 0.001$ compared with PBS, $n = 9 \pm$ standard deviation. Scale bars represent 50 µm.

2dDR: 2-Deoxy-ribose; E2: 17 β -Estradiol; LCA: Lens culinaris agglutinin; PBS: Phosphate-buffer saline.

3.3. Characterization of the E2 and 2dDR releasing PHBV scaffolds

3.3.1. Effect of including E2 and 2dDR on the ultrastructure of the PHBV scaffolds

SEM images of the E2 and 2dDR releasing PHBV scaffolds can be seen in Figure 4. The diameters of the fibres were significantly increased by addition of substances in all groups (25 mg E2 ($0.83 \pm 0.17 \mu\text{m}$), 50 mg E2 ($0.98 \pm 0.35 \mu\text{m}$), 250 mg 2dDR ($0.89 \pm 0.19 \mu\text{m}$), 500 mg 2dDR ($1.22 \pm 0.28 \mu\text{m}$)) added PHBV scaffolds when compared with the PHBV control group ($0.66 \pm 0.16 \mu\text{m}$) as shown in the graph on the bottom right corner of Figure 4.

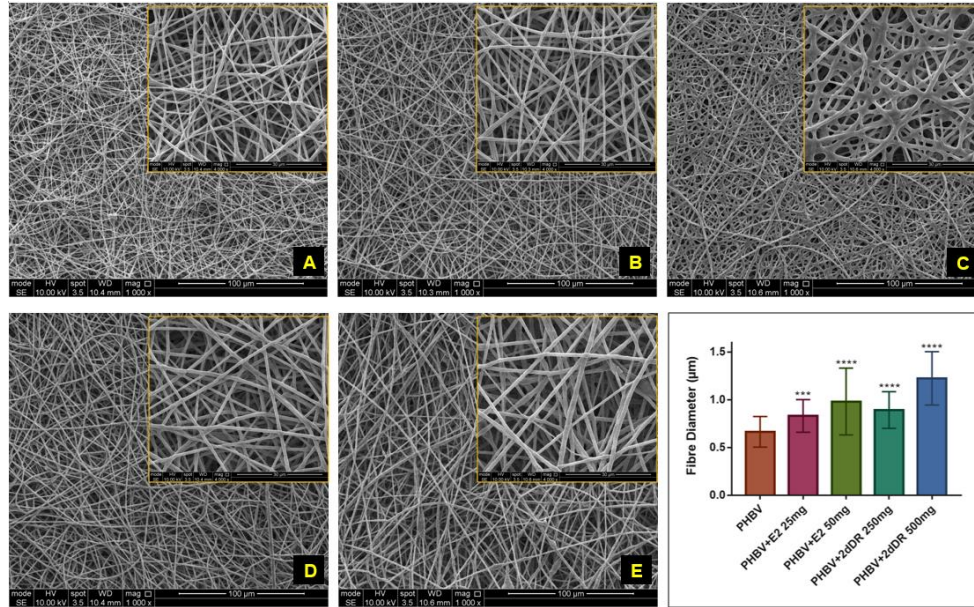


Figure 4. Scanning electron microscopy images of the scaffolds. (A) Plain PHBV, (B) PHBV + 25 mg E2, (C) PHBV + 50 mg E2, (D) PHBV + 250 mg 2dDR, (E) PHBV + 500 mg 2dDR. The graph on the bottom left corner shows the distribution of fiber diameters for each scaffold, **** $p \leq 0.0001$, *** $p \leq 0.001$. Scale bars represent 100 μm.

2dDR: 2-Deoxy-ribose; E2: 17β-Estradiol; PHBV: Poly3-hydroxybutyrate-co-3-hydroxyvalerate.

3.3.2. Release of E2 and 2dDR from PHBV scaffolds over 30 days

The rate of release of E2 and 2dDR from scaffolds was assessed over 30 days as shown in Figure 5A. By 7 days, 2dDR release from the scaffolds was 81.3% and 86.5% of the total 2dDR present in the polymer solution for 250 mg and 500 mg 2dDR scaffolds, respectively. In contrast the total E2 release from scaffolds within 7 days represented 1.3% and 1.6% of the initial E2 present in the polymer solution for 25 mg and 50 mg E2 scaffolds, respectively.

3.3.3. Comparison of the effects of E2 and 2dDR on the mechanical properties of scaffolds

When each scaffold was produced using the same volume of polymers, the thicknesses of the scaffolds were measured $105 \pm 22 \mu\text{m}$, $84 \pm 13 \mu\text{m}$, $62 \pm 10 \mu\text{m}$, $136 \pm 12 \mu\text{m}$, and $124 \pm 13 \mu\text{m}$ for plain, 25 mg E2, 50 mg E2, 250 mg 2dDR, and 500 mg 2dDR added PHBV scaffolds, respectively. As can be seen from Figure

5B, addition of all substances significantly increased the UTS of PHBV scaffolds when compared with plain PHBV scaffolds. Addition of E2 increased the UTS of the scaffolds more when compared with 2dDR added groups. Similarly, the stiffness of the scaffolds loaded with 2dDR and E2 was significantly higher compared to unloaded PHBV scaffolds. 250 mg 2dDR loading increased the stiffness of the PHBV scaffolds to the greatest extent as shown in Figure 5B. The wettability of the drug releasing scaffolds was investigated through a droplet retention time which is calculated using a drop shape analyser. Retention time of the water droplet on drug released scaffolds was calculated from recorded videos and given in Figure 5C. The water retention time on the 50 mg E2 loaded scaffolds increased to 198.3 ± 1.4 s from 16.7 ± 1.4 s observed for plain PHBV groups. In contrast, addition of 250mg 2dDR decreased the water retention time on scaffolds down to 2.3 ± 0.3 s. This showed that 2dDR loading made the scaffolds more wettable while the addition of E2 made the scaffolds less wettable.

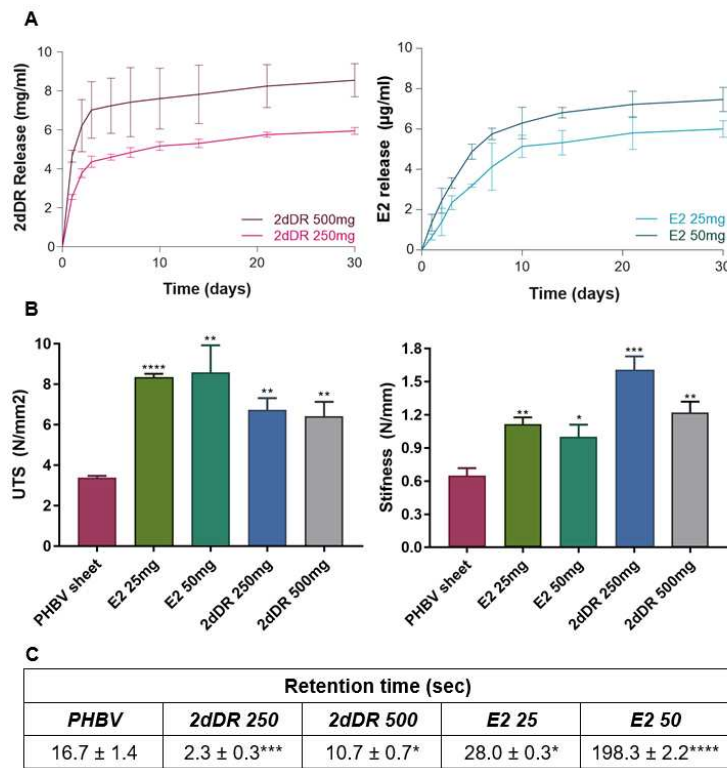


Figure 5. Assessment of the 2dDR and E2 release from the fibers over 30 days, and the effect of both drugs on mechanical properties of the scaffolds. (A) Release of 2dDR and E2 from PHBV scaffolds over 30 days, $n = 6 \pm$ standard deviation. (B) Comparison of UTS and stiffness and (C) droplet retention time on the scaffolds, **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, $n = 6 \pm$ standard deviation.

2dDR: 2-Deoxy-ribose; E2: 17β-Estradiol; PHBV: Poly3-hydroxybutyrate-co-3-hydroxyvalerate; UTS: Ultimate tensile strength.

3.4. Assessment of angiogenic potential of the E2 and 2dDR releasing scaffolds

Assessment of E2 and 2dDR releasing scaffolds on CAM demonstrated that all groups at least doubled the number of discernible blood vessels growing towards the scaffolds in comparison with plain PHBV

scaffolds as can be seen in Figure 6. Mean vessel counts for 25 mg E2 loaded scaffolds and 50 mg E2 loaded scaffolds were 49.5 (± 0.92) (**** $P \leq 0.0001$) and 37.9 (± 1.05) (**** $P \leq 0.0001$) respectively, while it was 48.6 (± 1.02) (**** $P \leq 0.0001$) and 37.1 (± 1.37) (**** $P \leq 0.0001$) for 250 mg and 500 mg 2dDR loaded scaffolds, respectively when compared with control groups (mean vessel count: 23.1 (± 1.24)). None of the loaded substances affected the embryo survival rate which was over 75% for each group.

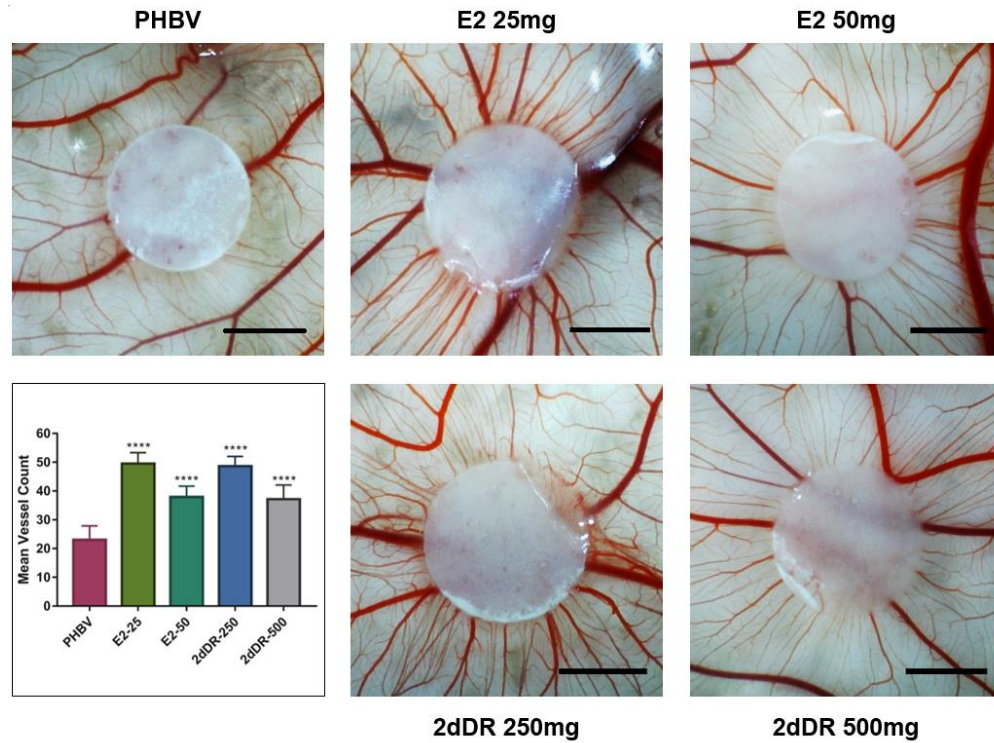


Figure 6. Representative images demonstrating the angiogenic potential of 2-dDR (250 mg and 500 mg) and E2 (25 mg and 50 mg) releasing scaffolds in comparison with PHBV scaffolds. The graph on the bottom left shows the quantitative data from these experiments-mean vessel counts, **** $p \leq 0.0001$. Scale bars represent 3 mm, $n = 6 \pm$ standard deviation.

2dDR: 2-Deoxy-ribose; E2: 17 β -Estradiol; PHBV: Poly3-hydroxybutyrate-co-3-hydroxyvalerate.

3.5. Histological analysis of the E2 and 2dDR releasing scaffolds on CAM

All groups showed good biocompatibility and similar changes in the structure of CAM with a small increase in cell density in the mesoderm layer in all scaffold groups. The mean number of blood vessels adjacent to the scaffolds was significantly increased in response to all concentrations of both E2 and 2dDR releasing scaffolds when compared with controls and CAM only groups (see Figures 7 and 8).

All scaffolds whether loaded with pro-angiogenic agents or unloaded showed good attachment to the CAM, and all membranes showed similar cellular infiltration. Figure 7 shows representative histology images.

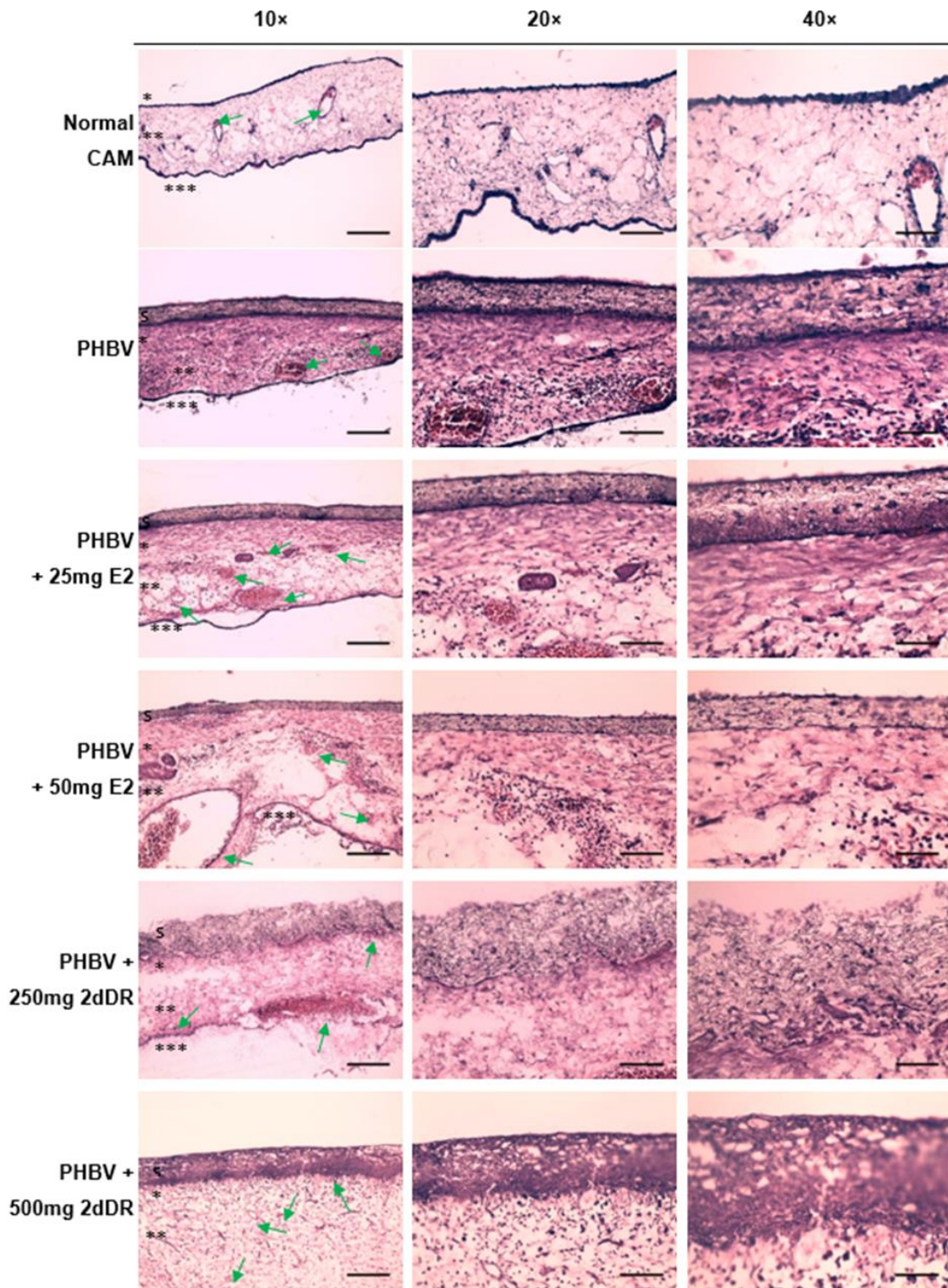


Figure 7. Histological analysis of chorioallantoic membranes after 7 days of incubation with or without scaffolds in different magnifications. Orientation of the scaffold(s), CAM ectoderm (*), mesoderm (**) and endoderm (***) layers were indicated in the images. Green arrows show the blood vessels. Scale bars = 0.2 mm (10x), 0.1 mm (20x), 0.05 mm (40x).

CAM: Chorioallantoic membrane; PHBV: Poly3-hydroxybutyrate-co-3-hydroxyvalerate.

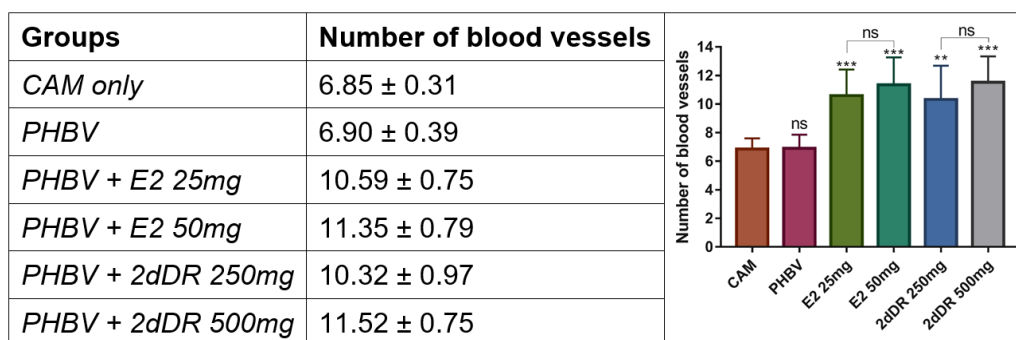


Figure 8. Quantification of the discernible blood vessels adjacent to the scaffolds at 10× magnification from the total of six different slides for each group and six different area of interest from each slide.

CAM: Chorioallantoic membrane; ns: Non significant; PHBV: Poly3-hydroxybutyrate-co-3-hydroxyvalerate.

4. DISCUSSION

As laboratory production of tissue engineered materials has progressed to the clinic it has become clear that one of the critical barriers to their success is the need to achieve rapid (within 5 days) neovascularisation post implantation. Rapid ingrowth and infiltration of blood vessels are crucial for biomaterials to be able to survive *in vivo* [54]. The need for improved neovascularisation is crucial for tissue engineering, and our laboratory has been seeking to develop biomaterials to promote angiogenesis. As already stated, the approach which has been the most investigated is to deliver the major proangiogenic growth factor VEGF, or more recently to co-deliver it with the naturally occurring glycosaminoglycan heparin to which it would normally be bound *in vivo*. The MacNeil group and many others have sought to deliver materials which bind heparin and are therefore attractive to the binding of VEGF [25,26]. However, introducing VEGF with the TE scaffolds requires multistep actions and a long process. Moreover, administration of large amounts of exogenous VEGF can lead to the formation of leaky and haemorrhagic vessels [15]. Thus, exploring new alternatives to the use of exogenous VEGF has critical importance.

Accordingly, we aimed to investigate the angiogenic potential of 2dDR and E2 either applied as solutions or released from TE constructs and explore that if it is possible to define certain concentrations of either 2dDR or E2 which are as effective as VEGF in stimulating angiogenesis in CAM assay. To date, there have been no studies conducted on the effective angiogenic dose range of 2dDR which is proangiogenic. Therefore, for the first time in this study, we demonstrated the reliable concentration ranges for both 2dDR and E2 for stimulating angiogenesis in CAM assay and compared their proangiogenic activities with the VEGF used as positive control.

The key findings from this study were that 2dDR and E2 showed dose-dependent angiogenic responses, and our results demonstrate that both were found approximately 80% potent for the production of new blood vessels *in vivo* when compared with VEGF as a positive control which proved to be highly angiogenic on the CAM. The results of the dose response studies showed that the most effective doses were 200µg/day/embryo and 200ng/day/embryo for 2dDR and E2, respectively. Higher and lower doses of the

drugs showed less angiogenic activity in the CAM assay. These results were further confirmed with the investigation of the microvascular structures of CAM when treated with the drugs. In VEGF treated groups, the VA% was far higher than in other groups. However, microvessels upregulated by VEGF showed smaller lacunae formation due to endothelial cell hypertrophy when assessed with confocal microscopy, while a more consistent and stable microvessel structure with a significantly increased VA% was found in 2dDR and E2 treated CAMs. This we suggest is important as VEGF has been reported to lead to the formation of leaky and haemorrhagic vessels [15]. Although angiogenic activities of 2dDR and E2 were evaluated with the direct applications of those to CAM, this administration method is only used for determination of the most angiogenic concentrations of the drugs.

The effective concentrations of 2dDR and E2 were then loaded into electrospun PHBV constructs to stimulate angiogenesis with the sustained release of both agents from fibres. PHBV is a natural, biocompatible and complete biodegradable biopolymer [55]. Although the biodegradability of PHBV is relatively slow when compared with other polymers like PLA and PCL [56,57], it is still an attractive material for drug release studies because of its excellent biocompatibility, biodegradability, easy processing properties [58].

Incorporation of 2dDR and E2 into the PHBV scaffolds resulted in some alterations in fibre morphology and hydrophobicity of the scaffolds although no technical problems or adverse effects of introducing E2 and 2dDR into fibres were experienced.

Introduction of both drugs significantly increased fibre diameters in accordance with the increasing concentrations of the drugs, and there appears to be a change in the fibre structure of electrospun PHBV scaffolds that contain higher concentrations of E2. Physical cross-linking of electrospun fibres (or fibre fusion) was observed in PHBV scaffolds loaded with the higher dose of E2. The fibre fusion can be explained with the decrease in viscosity of the electrospinning solution with the addition of E2, highly plasticizing agent [59]. This decrease in viscosity leads to slow evaporation of the solvent during electrospinning [60] which results in fused fibres. Unnithan et al. also reported similar changes in fibrous morphology of E2 loaded electrospun polyurethane (PU) scaffolds due to change of viscosity of the electrospinning solution [61]. On the other hand, the incorporation of 2dDR into the electrospun PHBV scaffolds did not affect fibre ultrastructure. However, although the SEM images did not show any change in fibre structure, we do observe reduced integrity of the fibres in histologic sections of 2dDR releasing scaffolds. These alterations occurred after the scaffolds were implanted on CAM. Therefore, it is likely that this change was due to rapid deformation of the fibres after the rapid release of 2dDR resulting in an increased surface area for cellular attachment and infiltration.

Addition of E2 significantly increased the hydrophobicity of the scaffolds in a dose dependent manner. This is not surprising as E2 is known to be highly hydrophobic [62]. In contrast to E2, the addition of 2dDR significantly decreased the hydrophobic character of the PHBV fibres which can be explained by the high solubility of 2dDR in water. The estimated solubility of E2 in water is approximately 30000 times lower than sugars [63,64]. Thus, the addition of these drugs with different solubilities significantly changed the hydrophilicity of the PHBV scaffolds. Furthermore, this had a reflection on the release characteristics. As can be expected, we observed significantly lower and slower release of E2 in comparison with the release

of 2dDR from the PHBV fibres. In contrast to the lower and more sustained release of E2 from PHBV fibres, a burst release of 2dDR was observed within 7-days.

Incorporation of both drugs enhanced both UTS and stiffness of the PHBV scaffolds compared with plain PHBV controls. The most interesting finding was that scaffolds with highest concentrations of E2 had the highest UTS and lowest stiffness. This is more likely to be due to the incorporation of E2 which is acting as a plasticizer within the electrospinning solution, changing the viscosity and leading to formation of larger diameter and fused fibres which could provide additional rigidity [65]. Huang et al. reported a similar change in tensile properties of their drug loaded PCL electrospun scaffolds. They demonstrated that the diameters of the fibres were increased with the addition of higher concentrations of the resveratrol (a kind of antioxidant), and the addition of the drug led to an increased ultimate strength and decreased stiffness of their scaffolds [66].

Although both of the factors stimulated angiogenesis when applied directly on CAM, a more dramatic increase in the number of discernible blood vessels was seen when 2dDR and E2 releasing PHBV scaffolds implanted on CAM. E2 was found to be slightly more effective in both application procedures when compared with 2dDR loaded and plain PHBV control groups. This study also established effective concentrations of E2 (25mg/g of PHBV) and of 2dDR (250mg/g of PHBV) to be loaded into scaffolds to achieve a reliable stimulation of neovascularisation. Higher doses of both drugs were found to be less effective in promoting the angiogenesis in CAM assay. The dose dependence of E2 has been studied by many groups. In 2004, Seo et al. reported E2 caused an increased angiogenic activity *in vivo* in a dose dependent manner [46]. Pence et al. studied the endogenous VEGF production of epithelial cells when treated with different doses of E2 *in vitro*. They found that the treatment of cells with 10 nM and 1000 nM E2 stimulated the production of VEGF where addition of 100 nM E2 showed no significant effect on cells [47]. Liu et al. examined the effect of E2 on rat cardiac microvascular endothelial cell (CMEC) proliferation and tube formation *in vitro*. With regards to the proliferation of CMECs, they reported a biphasic response to E2. The lowest and the highest doses of E2 were less effective compared to the optimal dose [48]. In contrast to studies of dosage response of E2, to date there have not been any such studies exploring the angiogenic response to different doses of 2dDR. In view of the comparatively few studies on this small sugar, not all of which agree, we conducted a dose dependence study of 2dDR for promoting angiogenesis. While lower doses of promoted angiogenesis, higher doses were found less effective. Drug releasing scaffold assays further confirmed these findings. The scaffolds loaded with the higher doses of 2dDR and E2 showed reduced angiogenic activity when compared with the scaffolds loaded with lower doses.

These results were then compared with the histologically stained sections of the scaffolds. In these, more blood vessels were counted adjacent to 2dDR and E2 releasing scaffolds. However, in contrast to the results from the quantified macro images, in histological analysis, the number of blood vessels adjacent to the scaffolds loaded with the higher doses of 2dDR (2dDR 500) and E2 (E2 50) were not significantly but slightly greater than lower doses. These differential results might be caused by our ability to see and count the smaller diameter blood vessels that may not be discernible in the digital CAM analysis used for quantification. Additionally, the histological sections also show blood vessels from different orientations

which might not have been detectable on digital CAM images. This might increase the results seen for higher doses of the drugs.

E2 has previously been reported as angiogenic both *in vitro* and *in vivo* by many groups as well as our research group. Albrecht et al. suggested that E2 promotes angiogenesis through up-regulation of VEGF. They reported rapidly increased VEGF expression and cell permeability by E2 administration to ovariectomized baboons [67]. Similarly, elevated VEGF mRNA expression levels were observed in ovariectomized rats after E2 treatment by Hyder et al. [68]. Morales et al. reported that E2 promoted migration of HUVECs and formation of capillary-like networks on matrigel [30]. Pence et al. indicated that exogenous E2 promoted endogenous production of VEGF by endometrial epithelial cells [47]. More recently our group has demonstrated release of E2 from both biodegradable PLA fibres [36] and from nondegradable PU fibres [69] with both electrospun scaffolds showing good proangiogenic activity in the CAM assay.

With respect to 2dDR, we recently published on a hydrogel containing this sugar that showed strong proangiogenic activity in the CAM assay and greatly accelerated wound healing in a rat skin model [45]. However, when it comes to the mechanism of action and the effective dose range of deoxy sugars, there is relatively little literature and it is contradictory. In 2010, Merchan et al. reported anti-angiogenic activity of 2-deoxy-D-Glucose (2-DG). They reported that, 2-DG inhibited endothelial capillary formation and endothelial cell migration *in vitro* [70]. In contrast there are a few studies which have reported 2dDR to be proangiogenic. Haraguchi et al. examined the angiogenic potential of the degradation products of thymidine on CAMs and found 2dDR induced angiogenesis in 72% of their eggs. They also reported its chemotactic activity *in vitro* and angiogenic activity *in vivo* [43]. Vara et al. recently published a detailed study exploring the mechanism of action of 2dDR in regulating angiogenesis. They found 2dDR induces angiogenesis by paracrine signalling. The 2dDR directly activates NADPH oxidase 2 (NOX2) which in turn triggers nuclear factor Kappa B (NF- κ B) which upregulates vascular endothelial growth factor receptor 2 (VEGFR2) and vascular endothelial growth factor-dependent angiogenesis [71]. Irrespective of the mechanism of action and dose response, our initial results looking at the response to hydrogels releasing 2dDR were very encouraging. 2dDR loaded chitosan (CS)/collagen hydrogels were firmly attached to the wound bed of full thickness excisional wounds (20 mm) in rats by day 3 and wounds treated with 2dDR, were completely closed by day 17 with clear hair growth while control wounds remained open with no evidence of epithelial tissue or hair growth. The histology of the wound beds of all animals sacrificed at day 17 showed complete healing in the case of 2dDR treated wounds with well-developed hair follicles and the presence of new blood vessels in the healed wounds was confirmed with CD34 staining [45].

In the assessment of these approaches to overcome delayed angiogenesis in tissue engineered constructs we have made extensive use of the *ex-ovo* CAM assay to evaluate angiogenesis. It is an excellent bioassay for assessing the angiogenic response to materials allowing direct imaging and comparison of the newly formed blood vessels. It can also be used as a low cost, rapid and simple tool for testing very early tissue reactions to biomaterials [72]. The CAM assay has been used by our group in recent years to evaluate the biocompatibility and proangiogenic response to a range of different polymers encompassing PLA, PU, PHBV, and CS/collagen.

To place the current results in context we summarise these preceding studies in a table which summarises the response of the *ex-ovo* CAM assay to these materials in terms of their effect on the structure of the underlying membrane, the infiltration of cells into the material and their effect on angiogenesis in the CAM. Cellular infiltration in the table describes the migration of cells from CAM to biomaterials. In general, highly porous, biocompatible structures where pore sizes are large enough to allow cellular ingrowth lead to good cellular/tissue infiltration to a biomaterial [73,74]. Because the diameter of the fibres and the pore size of electrospun PHBV were much smaller, less cellular infiltration was observed in all PHBV scaffold groups than for other polymers studied in the CAM assay (Table 1).

Table 1. Comparison of response of chorioallantoic membrane to different polymer systems, assessing biocompatibility and proangiogenic activity

Polymer	Functionalization	Average pore size	Effect on embryo survival	Cell infiltration	Angiogenic activity on CAM	Ref.
PLLA	None	<5 μm	None	++++	–	[36]
	Vitamin C		None	++++	++	
	E2		None	++++	++++	
PU	None	<25 μm	None	+++	–	[68]
	E2		None	+++	++++	
CS/PVA/PCL	Heparin	<1 μm	None	+++	++++	[26]
CS/PVA	Heparin	<6 μm	None	+++	++++	[27]
CS/Collagen	2dDR	<105 μm	None	+++	++++	[45]
PHBV	None	<3 μm	None	++	–	
	2dDR	<4 μm	None	++	+++	
	E2	<3 μm	None	++	++++	

While the current study makes a novel contribution to the literature on 2dDR by demonstrating a significant angiogenic response to different doses of 2dDR and 2dDR loaded PHBV scaffolds, there is much needed to be clarified about the angiogenic potential of this small deoxy sugar. We did not attempt to study the mechanism of action of 2dDR in promoting angiogenesis in this study. Our focus was to explore the dose response of 2dDR for promoting angiogenesis instead. With a biphasic dose-response curve, it raises the question of how different doses are acting in the promotion of angiogenesis. Higher or lower doses of the drugs can be not effective or even anti-angiogenic. That is why we did not achieve our primary objective of determining what concentration range of 2dDR is necessary for effective stimulation of angiogenesis, and how effective 2dDR is when compared to E2 and VEGF. That is why we did not achieve our primary objective of determining what concentration range of 2dDR is necessary for effective stimulation of angiogenesis, and how effective this is compared to E2 and VEGF. Although the CAM assay is a really useful assay for assessing the initial angiogenic response to biomaterials, the chick embryo does not have a fully developed immune system. Therefore, further animal studies will be needed to assess the inflammatory response to 2dDR (and E2) releasing PHBV scaffolds. In the current study, our main interest was to promote neovascularisation at the site of implantation by delivering drugs that would also potentially increase engraftment of TE constructs. We have now commenced further animal studies with 2dDR using a model of compromised wound healing. Beyond this, we will explore the engraftment and survival of tissue-engineered constructs by adding this sugar or incorporating it into the fibres of scaffolds prior to assembly of the tissue engineered construct.

5. CONCLUSION

We conclude that both direct application of 2dDR and E2 and the gradual release of these factors from PHBV fibres stimulated angiogenesis in an *ex-ovo* CAM assay. These two small stable factors were readily incorporated into electrospun fibres such as those that would be used for tissue-engineered constructs and have great potential to be used for functionalisation of the TE scaffolds to promote angiogenesis *in vivo*. Both gave results approximately 80% as effective as the addition of VEGF. In this study, the dose dependent angiogenic response to 2dDR has been demonstrated for the first time.

AUTHOR CONTRIBUTIONS

S Dikici contributed to the experimental design, analysis, acquisition and interpretation of data, statistical analysis, and drafting of this paper. N Mangir provided technical and material support to this work, and she contributed toward data processing and interpretation. S MacNeil, F Claeysens and M Yar contributed with their supervision and critical revision of the manuscript for important intellectual content.

SUMMARY POINTS

Background

- Delayed neovascularisation following implantation is a major problem for translation of tissue engineered (TE) constructs to the clinic.
- One approach is to combine a TE scaffold with a proangiogenic growth factor such as vascular endothelial growth factor (VEGF). VEGF is well-established as a critical factor in promoting angiogenesis *in vivo* but it is labile, expensive and rarely effective unless given at such high concentrations.
- In previous studies, angiogenic potential of E2 has been reported by our group and some other groups using different assessment methods. However, although there are many studies on dThdPase and assumptions on angiogenic activity of 2dDR as a degradation product by dThdPase activity, promoting angiogenesis with 2dDR still needs to be highlighted and supported by new studies.
- The present study was conducted to compare 2dDR, a novel and attractive proangiogenic small sugar, and E2, the primary female sex hormone, and to investigate their dose-dependent effects for the first time in stimulating angiogenesis in comparison with VEGF.
- This paper aims to form a basis for further understanding of angiogenic potential of 2dDR and to encourage scientific groups to explore this small, stable, and angiogenic agent in detail.

Results

- The angiogenic potency of 2dDR and E2 was initially assessed in an *ex-ovo* chick chorioallantoic membrane (CAM) assay using different quantification methods in order to validate the results. Both were found approximately 80% as potent as VEGF for promoting angiogenesis *in-vivo*.
- Furthermore, both agents were electrospun into PHBV fibres, and the sustained release of both agents from the scaffolds was observed over 30 days. Proangiogenic scaffolds stimulated neovascularisation over 7 days in the CAM assay.

Conclusion

- The results demonstrated that both direct application of 2dDR and E2 and the gradual release of these factors from PHBV fibres stimulated angiogenesis in an *ex-ovo* CAM assay.
- These two small stable factors were readily incorporated into electrospun fibres such as those that would be used for tissue-engineered constructs and have great potential to be used for functionalisation of the TE scaffolds to promote angiogenesis *in vivo*.

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