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**Estradiol releasing biodegradable mesh stimulates collagen production and angiogenesis:  
an approach to improving biomaterial integration in pelvic floor repair**

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## **Background**

Polypropylene meshes cause severe complications in some patients. Materials that are biomechanically compatible and can better integrate into host tissues are urgently needed.

## **Objective**

To design an estradiol-releasing electrospun poly-L-lactic acid (PLA) scaffold and evaluate its ability to stimulate new extracellular matrix and new blood vessel formation.

## **Design, setting and participants**

Human adipose derived mesenchymal cells (ADMSC) were from fat tissue. Electrospun PLA scaffolds containing 1%, 5% and 10% estradiol were constructed. and used for *in vitro* and *in vivo* experiments.

## **Interventions**

The angiogenic potential of the scaffolds was evaluated using an *ex ovo* chorioallantoic membrane (CAM) and chick aortic arch assays.

## **Measurements**

Estradiol release was measured fluorimetrically. The effect of scaffolds on proliferation and extracellular matrix (ECM) production of ADMSC was assessed by immunohistology. Mechanical properties were tested using a tensiometer.

## **Results and limitations**

Scaffold structure was not affected by the inclusion of estradiol and mechanical properties were only slightly modified. Estradiol was released from PLA scaffolds over a 5-month period. ADMSCs cultured on estradiol-releasing PLA scaffolds produced more ECM involving collagen I, III and elastin. Estradiol-releasing scaffolds doubled new blood vessel formation in the CAM assay ( $p= 0.001$ ) and outgrowth of pro-angiogenic cells in the aortic arch assay ( $p= 0.001$ ). Further *in vivo* studies in higher animal models are required.

## **Conclusion**

Estradiol releasing PLA scaffolds increase ECM production and stimulate angiogenesis. As such, they are promising candidate materials to be used in pelvic floor repair and to improve the initial healing phase of a repair material following implantation.



## **Patient summary**

In this study we designed a tissue engineered material that can be used to support weakened pelvic floor tissues in women avoiding the complications associated with current surgical mesh. Our results showed that this material can integrate well into tissues by stimulating new blood vessel formation.

## 1. Introduction

In the 1990s, Petros *et al* reported the implantation of PPL mesh tapes to treat stress urinary incontinence (SUI) <sup>1</sup>. Following this, larger areas of mesh were used to repair pelvic organ prolapse (POP), however, serious complications have now been demonstrated in up to 15% of women <sup>2</sup> and to a lesser degree in SUI surgery <sup>3</sup>. In 2016, the US Food and Drug Administration (FDA) re-classified trans-vaginal mesh devices from class II to III <sup>4</sup>. This led to a reduction in the number of trans-vaginal mesh surgeries performed for POP from 27% to 2% <sup>5</sup>, with a more modest decline in the number of mid-urethral tapes inserted <sup>6</sup>. It appears that we are now back to a pre- mesh era with some important lessons to be learned.

PPL devices evolved over many years from an initial metal wire mesh <sup>7</sup> to the current monofilament, macroporous mesh used in contemporary practice <sup>8</sup>. PPL mesh was not designed evaluated for safety in the pelvic floor prior to its introduction. The mechanical properties, microbial flora and vascularization status of paravaginal tissues are very different to that of the abdomen. Recent data has demonstrated site-specific responses to implanted PPL mesh; contraction and exposure of the mesh occurred following trans-vaginal implantation in sheep, while no complications were demonstrated following abdominal implantation over 12 months <sup>9</sup>. Furthermore, PPL mesh undergoes an M1 (proinflammatory) macrophage response, characterized by expression of pro-inflammatory cytokines, which leads to a vigorous foreign body reaction <sup>10</sup>.

Materials that are specifically designed for implantation into the pelvic floor are urgently required <sup>11</sup>. In exploring several candidate materials, we identified electrospun poly-L-lactic acid (PLA) scaffolds as a material, which possess mechanical properties more closely related to those of healthy paravaginal tissues <sup>12</sup> and it induces an M2 macrophage response associated with constructive remodelling <sup>10</sup>. Electrospun PLA may therefore lead to better integration during the initial healing phase following implantation, whilst providing appropriate structural support for tissues.

A well vascularized wound bed is crucial to achieve rapid integration of materials following implantation. Clinical studies report a decrease in vaginal blood flow in postmenopausal women, which improves significantly after estrogen replacement <sup>13</sup>. 17- $\beta$  estradiol plays a vital role in maintenance of normal structure and function of pelvic tissues. It is an inhibitor

of matrix metalloproteinases <sup>14</sup> and stimulates neo-collagen synthesis <sup>15</sup> and directly stimulates vascular endothelial cells through the estrogen receptor <sup>16</sup>.

A potential solution to improving the angiogenic potential of repair devices is to incorporate pro-angiogenic drugs into materials <sup>17</sup>. The aim of this study was to develop an estradiol-releasing electrospun mesh of PLA and evaluate its ability to support cell proliferation, and stimulate neovascularization to improve the integration of materials into the wound bed suitable for urogynaecological applications.

## **2. Materials and Methods**

### ***2.1. Electrospinning***

Poly-L-lactic acid (Goodfellow, Cambridge, UK) at 10% (wt/v) was dissolved in dichloromethane. 17- $\beta$ estradiol (Sigma-Aldrich) was added at 1%, 5% and 10% by mass of polymer. 20mls of polymer solutions were loaded into syringes fitted with 21G needles, placed into a syringe pump (Genie<sup>TM</sup> Plus, Kent Scientific, USA), and delivered at 40 $\mu$ l/min per syringe. Random microfibres were created at an accelerating voltage of 17kV DC and collected on an aluminium foil covered earthed mandrel rotating at 300rpm.

### ***2.2. Scanning Electron Microscopy (SEM)***

Samples were mounted on 12.5mm stubs and sputter coated with 25nm of gold (Edwards sputter coater S150B, Crawley, England). SEM (Philips/FEI XL-20 SEM; Cambridge, UK) was performed at an accelerating voltage of between 10-15 kV and a SPOT size between 2 and 3. Fibre diameter and pore size were measured using ImageJ software (National Institute of Health)<sup>18</sup>.

### ***2.3. Release of estradiol from scaffolds***

Scaffolds were cut to 15mm<sup>2</sup>, weighed and incubated in 1ml/well of Phosphate buffered saline (PBS). The estradiol concentration released from each of the 3 scaffold groups (10mg (1% wt/vol), 50mg (5% wt/vol) and 100mg (10% wt/vol)) over 5 months was measured fluorimetrically (Kontron SFM 25 spectrofluorimeter) at  $\lambda_{ex}$ 277nm/ $\lambda_{em}$ 310nm against solutions of known concentration (n=6 per group). Fresh PBS was replaced after each sample was removed.

### ***2.4. Culture of Adipose-derived mesenchymal stem cells (ADMSC) on scaffolds***

ADMSC were cultured from human subcutaneous fat as described previously<sup>12</sup>, donated under an HTA research tissue bank license (08/H1308/39). Cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Advanced Protein Products, Brierley Hill, UK), 2mM glutamine, 0.625 $\mu$ g/mL amphotericin B, 100IU/mL penicillin and 100 $\mu$ g/mL streptomycin (Gibco Invitrogen, Paisley, UK).

Scaffolds were cut to 15mm<sup>2</sup> and sterilized in 70% ethanol. 500,000 passage 3 ADMSC were seeded per scaffold and samples cultured for 2 weeks at 37°C, 5% CO<sub>2</sub>. The metabolic

activity of seeded cells was quantified by AlamarBlue™ (Sigma-Aldrich, Dorset, UK) assay at days 7 and 14, as described previously<sup>18</sup>.

### ***2.5. Assessment of ability of ADMSC to proliferate and produce extracellular matrix on estradiol releasing scaffolds***

Samples were fixed with paraformaldehyde after 2 weeks of culture and stained with 1ml of 1ng/ml of 4',6-diamidino-2-phenylindole dihydrochloride ((DAPI) Gibco Invitrogen, Paisley, UK). After incubating with bovine serum antigen, primary antibodies were added, including goat anti-human collagen I, III and rabbit anti-human alpha elastin (AbD Serotec, Oxford, UK). 100µl of 1:20 primary and 100µl of 1:50 secondary antibodies were added, including FITC labelled anti-goat IgG and anti-rabbit IgG.

DAPI stained nuclei and FITC labelled matrix components were imaged using a fluorescent microscope (Axon ImageXpress™, Molecular Devices Limited, Union City, CA) by switching between filters for DAPI ( $\lambda_{ex}$ 385nm/ $\lambda_{em}$ 461nm) and FITC ( $\lambda_{ex}$ 495nm/ $\lambda_{em}$ 515nm).

Total collagen production was measured by Sirius Red staining as described previously using 0.1% solution of Sirius Red F3B in saturated picric acid<sup>18</sup>.

### ***2.6. Assessment of effects of estradiol on differentiation capacity of ADMSC***

ADMSCs at passage 3 were cultured with 15mm<sup>2</sup>, weight matched sections of either estradiol-releasing or control PLA scaffolds in DMEM (n=6). After 24 hours, media was replaced with specific induction medium and cultured for 3 weeks. Adipogenic and osteogenic induction media contained the following supplements in addition to above defined DMEM: 1 µM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 µg/mL insulin and 100 µM indomethacin for adipogenic media and 10 nM dexamethasone, 50 µg/mL ascorbate-2-phosphate and 2 mM β-glycerophosphate for osteogenic media.

Cells were fixed and stained with Oil Red O and Alizarin Red for adipogenic and osteogenic phenotypes, respectively. Counter staining was performed using haematoxylin. Histological differences were assessed blindly by 6 post-doctoral researchers. All reagents were from Sigma-Aldrich, Dorset, UK.

### ***2.7. Assessment of effect of estradiol on mechanical properties of scaffolds***

Scaffolds were cut into 5mm x 10mm sections and clamped into a tensiometer (BOSE

Electroforce Test Instruments, MN) using a 22N load cell and a ramp test at a rate of 0.1mm/sec. Results were standardized by width and thickness of materials (N/mm<sup>2</sup>). Tests were performed both before and after 7 days of ADMSC culture on the scaffolds.

The initial linear gradient of each resulting stress vs strain curve was taken as the Young's modulus (N/mm<sup>2</sup>), while the ultimate tensile strength (UTS) was taken as the force per area of sample at break/maximal point. Both values were compared to data published for paravaginal tissues of healthy premenopausal patients <sup>19</sup>.

### ***2.8. Assessment of angiogenic potential of estrogen releasing scaffolds***

Angiogenesis was assessed using an *ex ovo* chick chorionic allantoic membrane (CAM) and an *in vitro* chick aortic arch assay. Pathogen-free fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Henry Stewart Co. (UK). Care was consistent with the Home Office, UK guidelines. Embryos were cultured using an *ex ovo* (shell less) method <sup>20</sup>. Embryos were transferred into a petri dish on embryonic development day (EDD) 3 and maintained in a humidified incubator at 38°C between EDDs 3 to 14. Embryo survival was assessed daily.

#### *The ex ovo CAM assay*

Scaffolds were cut into 6.5mm diameter circles using an Epilog Laser Cutter (Clevedon, UK) and sterilized under UV light. At EDD 8, two scaffolds were placed on the CAM surface. At EDD 14, images of scaffolds and surrounding CAM area were taken with a digital microscope. After imaging, 0.1mL of a 20% solution of Lens culinaris agglutinin [LCA (Vector Laboratories)] was injected into the circulation using a 30G hypodermic needle to fluorescently label intraluminal endothelial cells. Embryos were sacrificed and scaffolds were removed from the CAM surface. Angiogenesis was quantified by counting all discernible blood vessels traversing a 1mm annulus about the 2mm imaginary circle drawn around the scaffold <sup>21</sup>.

#### *Chick aortic assay*

Embryos were sacrificed on EDD 14, the aortic arch removed and cut into 1mm rings under a stereomicroscope. Aortic rings were embedded in 50µL of Matrigel® (Basement Membrane Matrix, Corning®) and cultured in Minimum Essentials Media supplemented with

2.5% FCS, 50 units/mL penicillin and 50 $\mu$ g/mL streptomycin (GIBCO, Carlsbad, CA). Rings were co-cultured with scaffolds using an insert (Greiner Bio-One GmbH). Endothelial sprout length was observed under an inverted microscope from day 5. Endothelial cells were characterized by immunofluorescence staining with Isolectin B4 (Vector Laboratories, Burlingame, USA).

## **2.9. Histology**

Scaffolds and surrounding CAM were fixed with paraformaldehyde, and embedded in freezing medium. 6 $\mu$ M sections were taken using a cryostat (Leica CM1860UV, Leica, Germany). Slides were stained with DAPI and observed under an epifluorescence microscope (Olympus, Japan).

## **2.10. Statistics**

Statistical differences between two group means were analysed using Student's T test when the data was normally distributed and with Mann Whitney U test otherwise. Survival of the chick embryos were analysed with the Kaplan Meier method. A p value of <0.05 was considered statistically significant.

### **3. Results**

#### ***3.1. Effect of estradiol on scaffold ultrastructure***

There was no significant difference between the fibre diameter and pore size of PLA versus estradiol-releasing PLA scaffolds ( $0.65\pm 0.30\ \mu\text{m}$  vs  $0.71\pm 0.28\ \mu\text{m}$  [ $p=0.47$ ] and  $4.26\pm 2.29\ \mu\text{m}$  vs  $4.25\pm 2.04\ \mu\text{m}$  [ $p= 0.98$ ], respectively) as shown in Figure 1

#### ***3.2. Release of estradiol from scaffolds over 5 months***

The cumulative release of estradiol increased for each time-point, until no further estradiol was released as demonstrated in Figure 2. The total estradiol released from the scaffolds was equivalent to 2.5%, 1.4% and 2.45% of the initial estradiol present in the polymer solution for the 10mg, 50mg and 100mg estradiol scaffolds, respectively. A burst release between 40- 50% of the drug was observed in the first 14 days.

#### ***3.3. Effect of estradiol releasing scaffolds on differentiation capacity of ADMSC***

Culturing ADMSC in specific induction media resulted in cellular differentiation over 3 weeks, including the formation of lipid vesicles for ADMSC cultured in adipogenic medium and the presence of calcium for cells cultured in osteogenic medium (Figure 3). There were no significant differences in cell morphology, lipid vesicles or calcium content between control and estradiol-releasing scaffolds, as assessed using blind scoring.

#### ***3.4. Effect of estradiol on metabolic activity and extracellular matrix production of ADMSCs***

Figure 4a demonstrates an increase in metabolic activity from 7 to 14 days for cells cultured on both estradiol and control scaffolds, with a greater increase seen for cells cultured on estradiol scaffolds ( $p<0.01$ ). ADMSCs cultured on estradiol scaffolds for 14 days produced a greater proportion of collagen than cells cultured on control scaffolds ( $p<0.001$ ) as demonstrated in Figure 4b. Figure 4c shows a greater proportion of collagen I and III and elastin from culture of ADMSC on estradiol scaffolds compared to controls.

#### ***3.5. Effect of estradiol on the mechanical properties of scaffolds***

Figure 5a demonstrates that estradiol-releasing scaffolds show a Young's Modulus closer to the values of healthy native fascia. After 2 weeks of cell culture, both scaffolds become more elastic, however estradiol-releasing scaffolds were significantly stiffer ( $p<0.01$ ) than



control scaffolds. Figure 5b demonstrates that estradiol-releasing scaffolds are significantly stronger than controls, both before and after 2 weeks of cell culture. Of note, the UTS of control scaffolds decreases after 14 days of cell culture, while the UTS of estradiol-releasing scaffolds increases.

### ***3.6. Effect of estradiol on the angiogenic properties of PLA scaffolds***

The CAM assay demonstrates at least double the number of blood vessels growing towards the estradiol scaffolds compared to controls over 7 days (mean vessel count: 25.0 ( $\pm 5.29$ ) and 10.67 ( $\pm 2.64$ ) respectively,  $p < 0.001$  as shown in Figure 6A and B). Estradiol did not affect embryo survival rates (Figure 6C).

Estradiol-releasing PLA scaffolds induced longer and denser endothelial sprouts when co-cultured with chick aortic rings (Figure 7A). Figure 7C demonstrates a sprout length/radius of 1.6 ( $\pm 0.51$ ) and 1.0 ( $\pm 0.44$ ) for estradiol-releasing and control scaffolds respectively ( $p = 0.01$ ) (Figure 7C).

Host tissues infiltrated into each scaffold to a similar degree (Figure 8). There was an increased number of blood vessels (stained with fluorescent angiography) in the CAM tissue adjacent to and in- between the fibres of the estradiol-releasing scaffolds compared to controls.

#### 4. Discussion

Biodegradable repair materials should integrate well into the patient's tissues and be strong enough to provide structural support, while regeneration of host tissues over time eventually assumes this load bearing function.

In this study we demonstrate that incorporation of estradiol into the fibres of an electrospun PLA scaffold do not significantly alter the overall microstructure and mechanical properties of the scaffold. Tissue integration was assessed in this study through the interaction cells *in vitro* with the scaffold and the ability of the scaffold to stimulate angiogenesis *in vivo*. For the former, ADMSC were selected as a representative cell source, capable of undertaking a range of phenotypes. Estradiol was released over a five-month period in a dose-dependent manner. Scaffolds consisted of random micro-fibres with a range of pore sizes up to 10µm. While porosity influences the ease of cell infiltration into the scaffold, our previous studies with electrospun PLA implanted in rats<sup>22</sup> and rabbits<sup>10</sup> demonstrates a rapid integration of host tissues with an M2 macrophage (tissue remodelling) response.

Several assessments of non-degradable polypropylene meshes in dogs<sup>23</sup>, sheep<sup>9</sup>, rhesus macaque<sup>24</sup> and humans<sup>25</sup> demonstrate sustained chronic inflammation. Biodegradable PLA meshes last intact for at least one year when implanted in rat models<sup>22</sup>. In the current study we demonstrate that inclusion of estradiol improved the ability of ADMSCs to stimulate new ECM production including collagen I, III and elastin.

The most critical feature of this estradiol-releasing PLA scaffold is the significant increase seen in the number and density of blood vessels and integration of tissues into this material when implanted into the CAM assay. The CAM assay is an established method for the *in vivo* evaluation of angiogenesis, which allows direct visualization of the newly forming vessels into a biomaterial<sup>26</sup>.

An estradiol releasing electrospun scaffold has previously been described for use as a wound care product in post-menopausal women, where the anti-inflammatory properties of estradiol were utilized<sup>27</sup>. Estradiol has been demonstrated to stimulate endothelial cells to produce VEGF when released from a collagen based scaffold<sup>28</sup>. Here we demonstrate for the first time that estradiol released from an electrospun scaffold dramatically stimulates

new blood vessel formation in and around the material. Although we have designed this material for use in pelvic floor repair, it could have wider applications in all areas of tissue engineering as a pro-angiogenic scaffold.

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## \*Take Home Message

Surgical mesh used in pelvic floor is associated severe complications in some patients. There is an unmet and urgent need for better materials. Tissue engineered scaffolds that can better integrate into host tissues can be designed making use of estradiol.

Figures

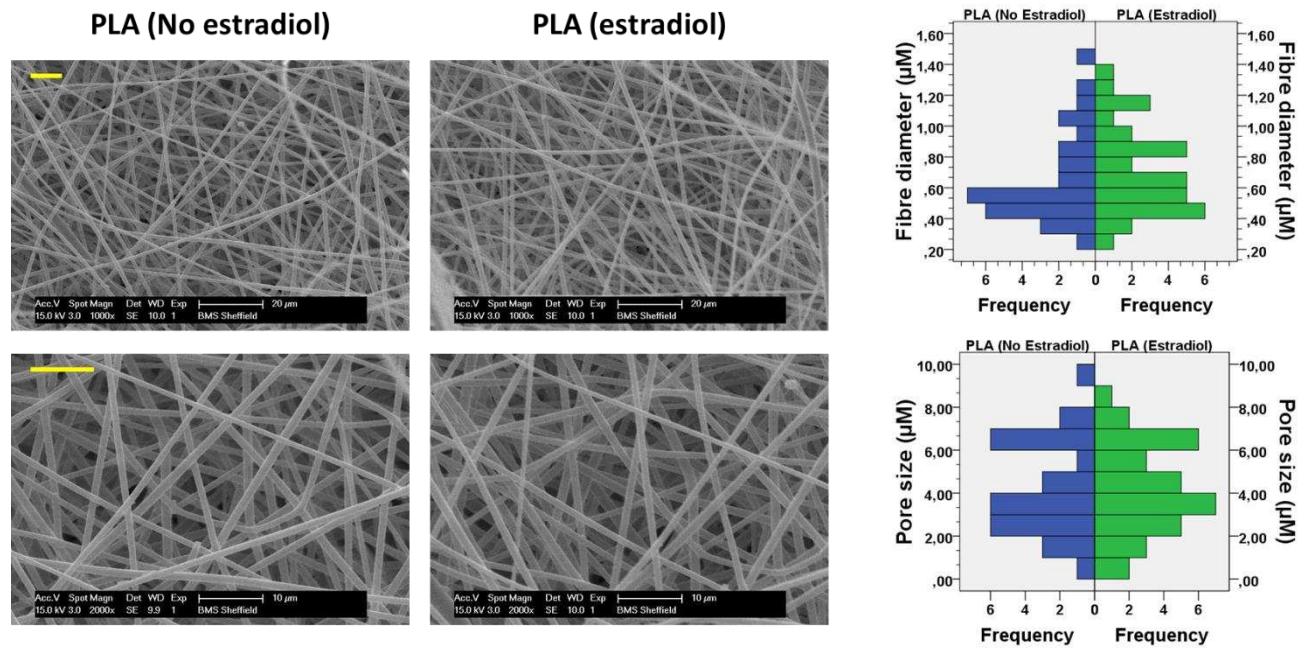


Figure 1. Representative images of scaffolds under SEM. Histograms on the right demonstrate distribution of fibre diameters and pore sizes in both scaffolds. Scale bars represent 10 µm.

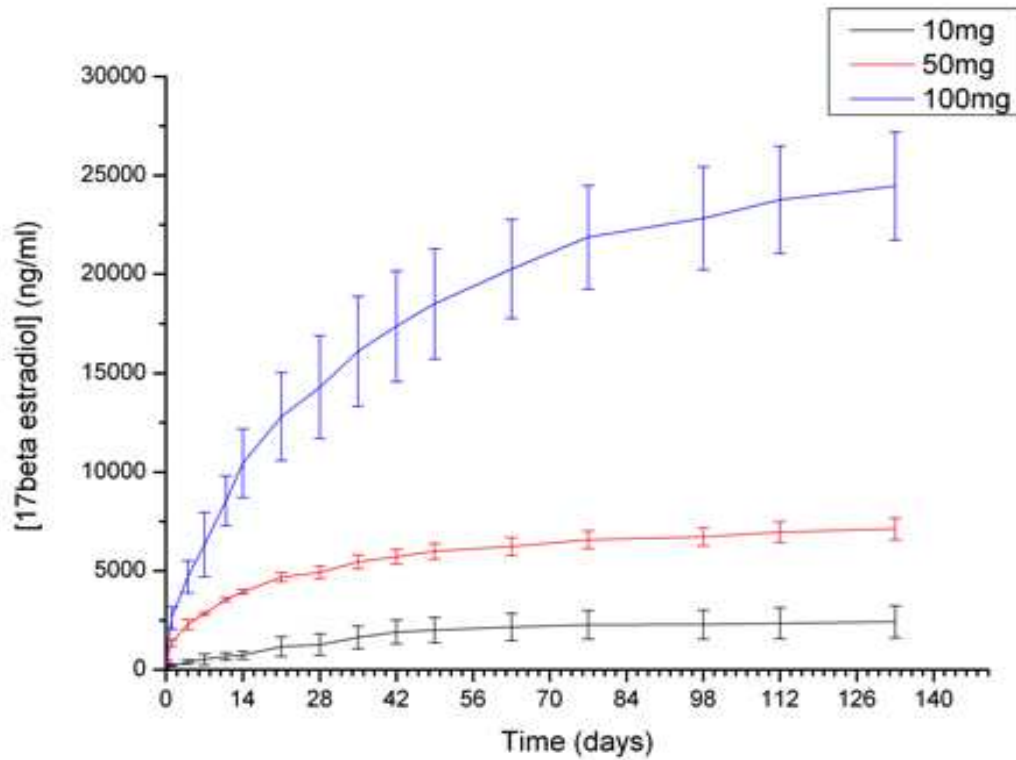


Figure 2. Cumulative release of estradiol from 3 different estradiol releasing scaffolds over 133 days as measured using a spectrofluorometer ( $n=6\pm SEM$ ).

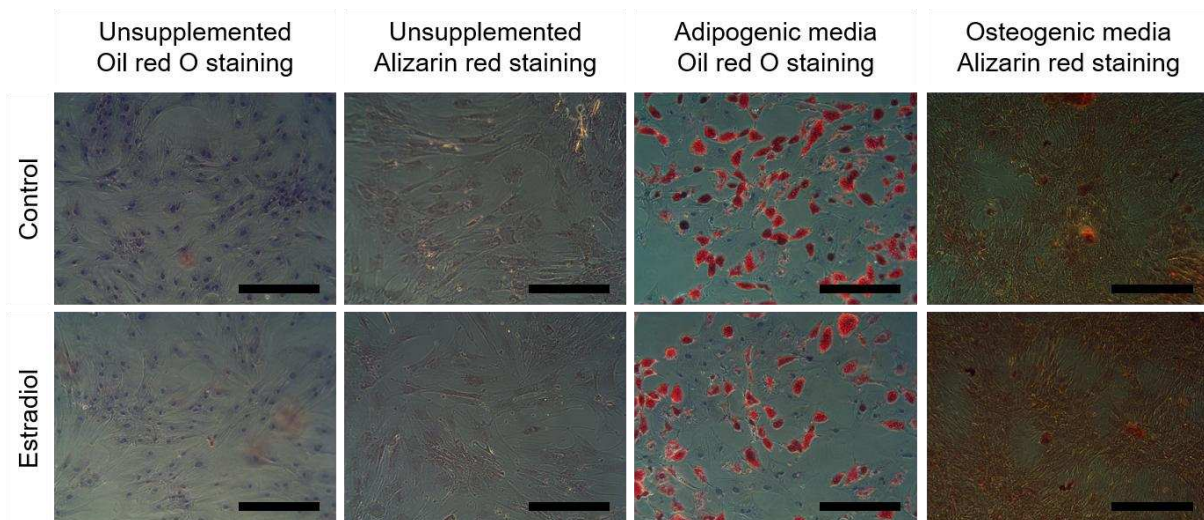
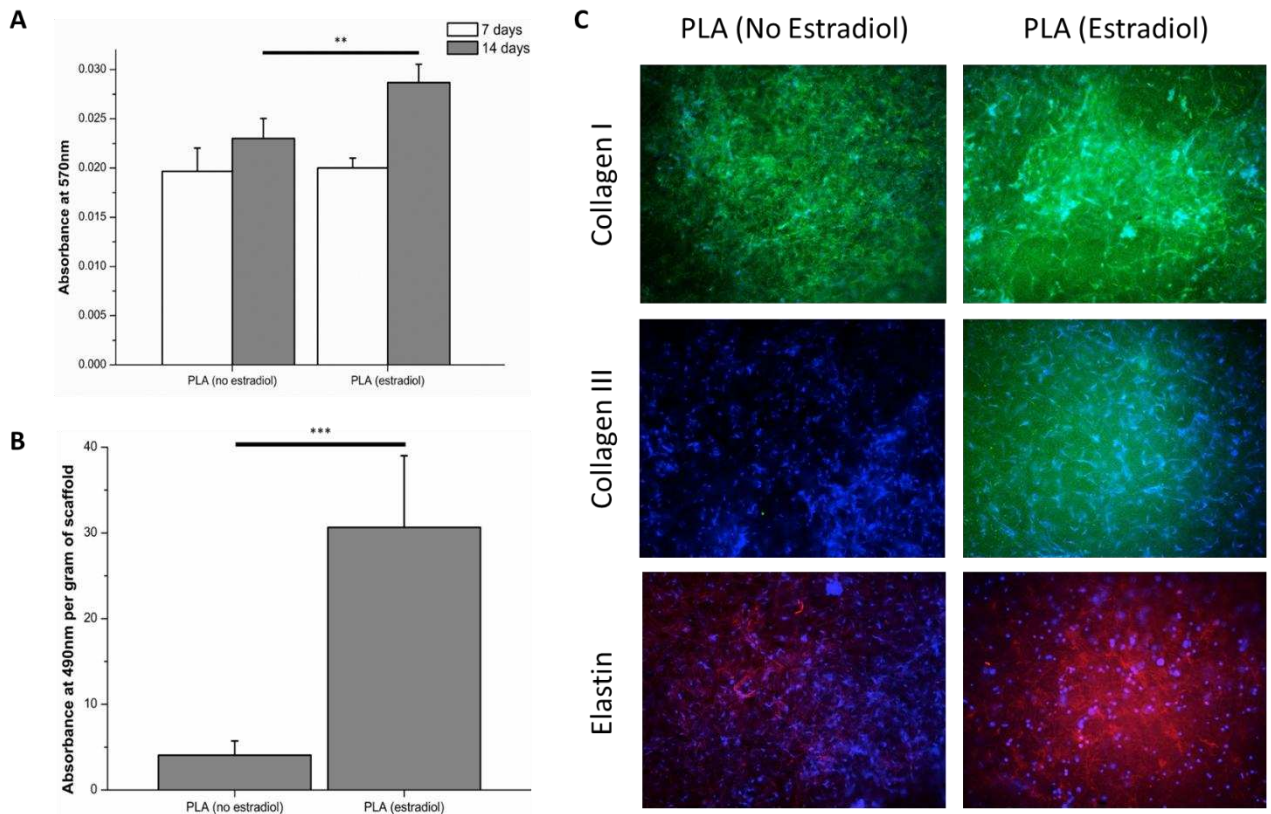


Figure 3. Differentiation assays. ADSC cultured either in DMEM, adipogenic media or osteogenic media, stained with haematoxylin and Oil red O (for lipid vesicles) or Alizarin red (for calcium). Scale bar represents 50 $\mu m$ .





**Figure 4.** Extracellular matrix production by ADSC cultured on Estradiol releasing PLA scaffolds compared to PLA only scaffolds. (A) Cell metabolic activity of ADSC cultured on control and estradiol releasing PLA scaffolds as measured by AlamarBlue assay with absorbance measured at 570nm using plate reading colourimeter. ( $n=6 \pm SEM$ ).  $**p < 0.01$ . (B) Total collagen production at day 14 as measured by Sirius red assay with absorbance measured at 490nm using plate reading colourimeter. ( $n=6 \pm SEM$ ).  $***p < 0.001$ . (C) Fluorescent microscopy images of extracellular matrix components produced by ADSC after 14 days of culture. Collagen I (green), elastin (red) and cells (blue). Scale bar represents  $100\mu m$ .

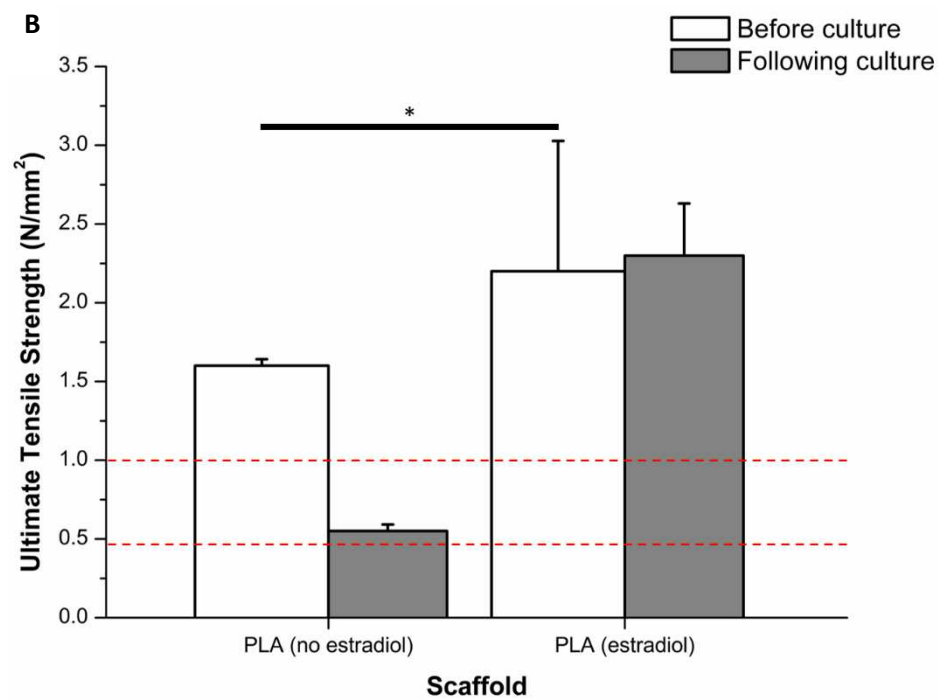
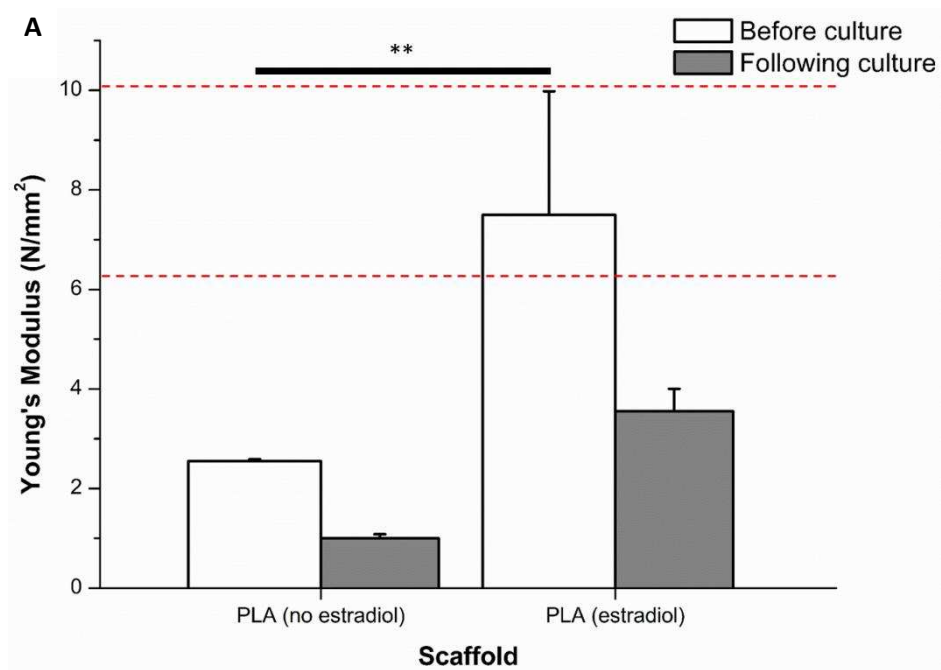


Figure 5. The effect of oestradiol on the strength of the scaffolds assessed in the presence of ADMSCs.

A. Young's Modulus of control and estradiol releasing PLA scaffolds, before and after 14 days of cell culture with ADSC. Area between dashed lines represents values for healthy native fascia [18].

$N=6\pm SEM$ ,  $**p<0.01$ .

B. Ultimate Tensile Strength of control and estradiol releasing PLA scaffolds, before and after 14 days of cell culture with ADSC. The area between the dashed lines represents values for healthy native fascia [18].  $N=6\pm SEM$ ,  $***p<0.001$ .

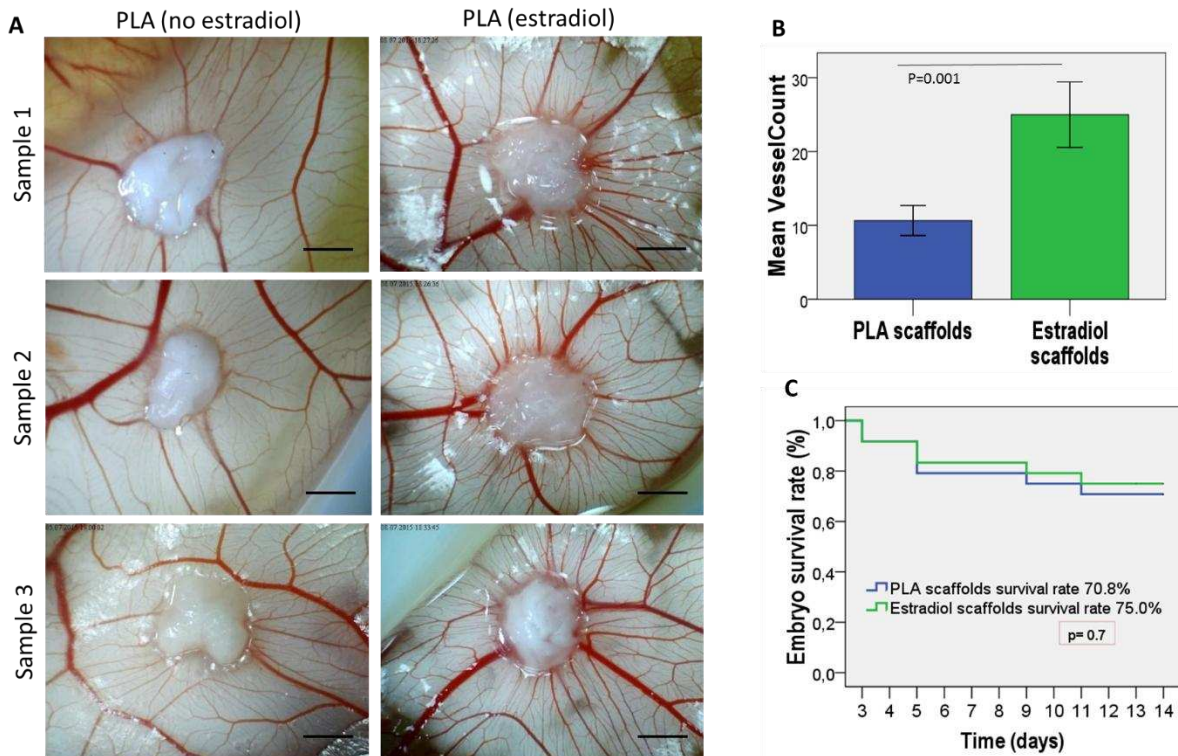
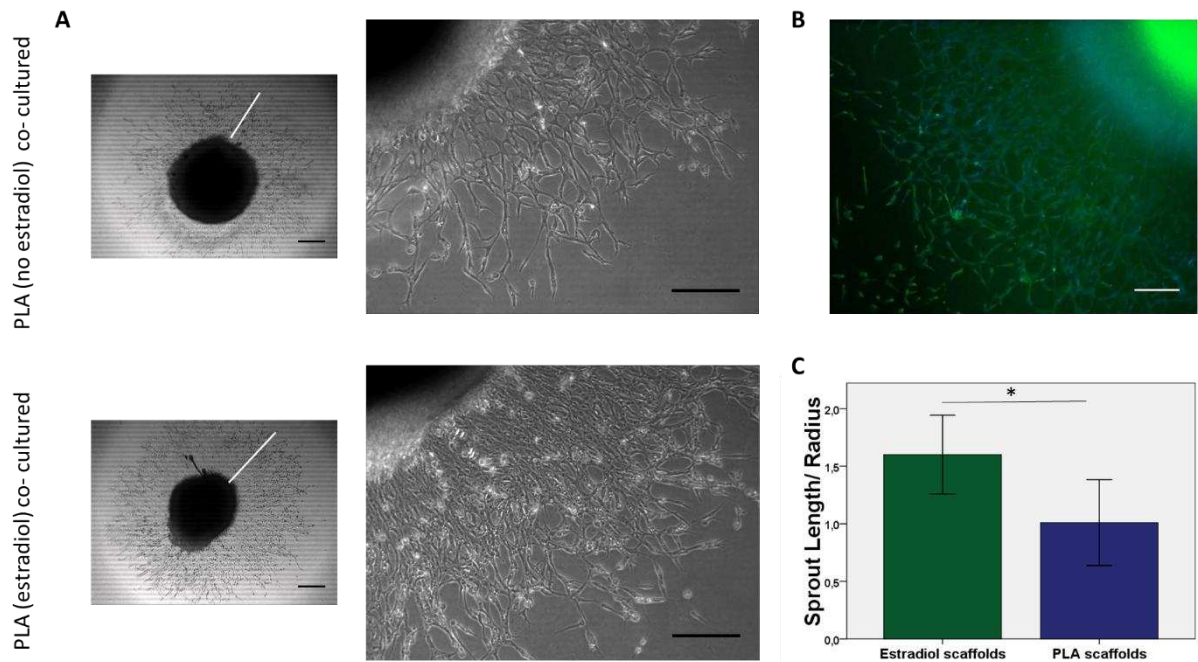
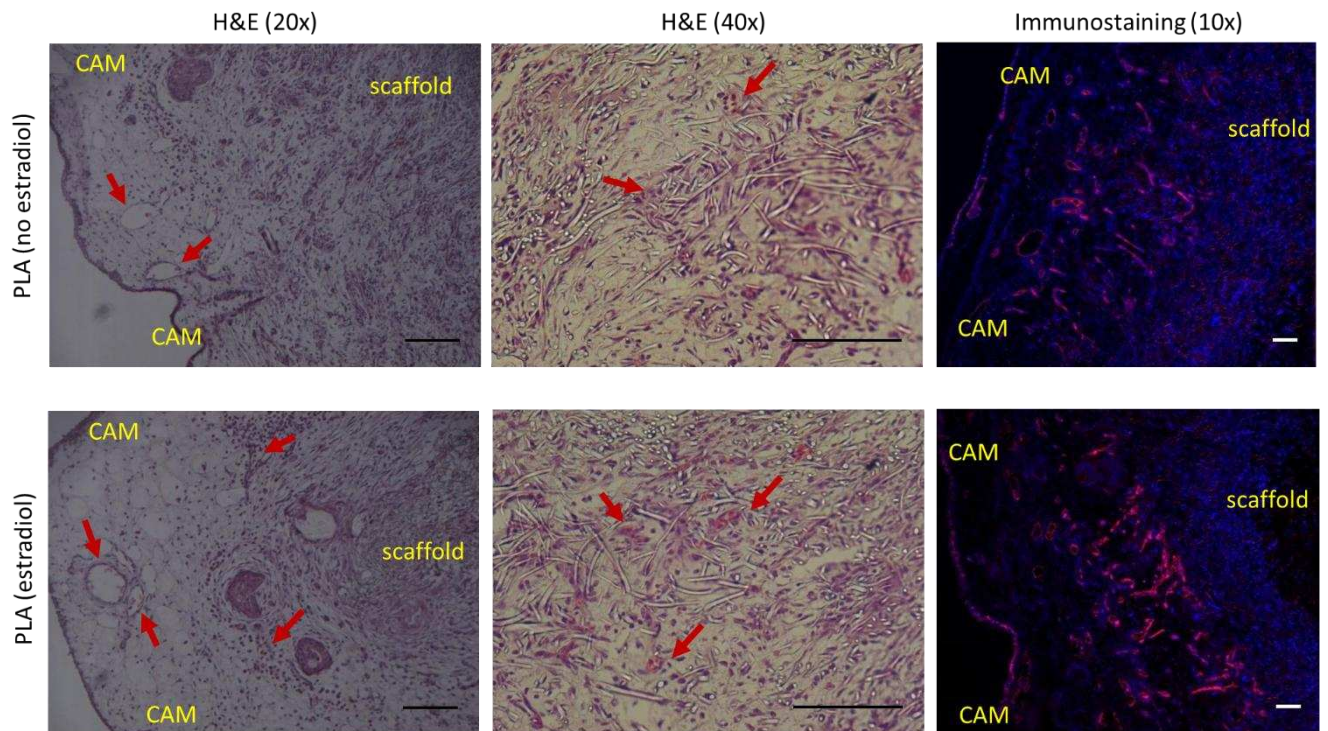


Figure 6. Angiogenic potential of estradiol releasing PLA scaffolds compared to PLA only scaffolds, day 14 on chicken chorioallantoic membrane (CAM) assay. A normal distribution of blood vessels was observed when the control PLA scaffold was placed on the CAM. With the estradiol releasing scaffold newly formed blood vessels can be seen to radiate towards the scaffold in a spoke-wheel pattern (A). The mean vessel count of estradiol releasing scaffolds was double that of the PLA scaffolds (B) and the embryo survival rate was the same between the two groups (C). (Scale bars represent 3mm).



*Figure 7. A) Evaluation of effect of estradiol on endothelial cell proliferation and sprouting. One millimetre rings from the chick aorta were embedded in Matrigel and co-cultured with PLA only scaffolds (upper row) or Estradiol releasing PLA scaffolds (lower row). The extent of endothelial sprouting by 5 days in culture is much greater in the presence of estradiol. B) Fluorescent microscopy images of immunological staining. Lectin IB4 - endothelial cells stained green, nuclear components stained blue (DAPI). C) Sprout length/radius results taken from 11A, \* P=0.01. (Scale bars represent 50  $\mu$ m).*



*Figure 8. A mild inflammatory reaction together with a normal distribution of blood vessels on the CAM adjacent to the PLA scaffold (upper row) can be observed compared to a significantly increased number of large blood vessels in response to Estradiol releasing PLA scaffold (lower row) at day 14, Haematoxylen & Eosin (H&E) staining (left side). Also on higher magnification several small blood vessels could be observed in between the PLA fibers which was more abundant in the presence of Estradiol (middle). On the right endothelial cells lining all sizes of blood vessels appear stained Rhodamine- conjugated Lens culinaris agglutinin and cell nuclei are stained with DAPI. Scale bars represent 100  $\mu$ m.*

**Estradiol releasing biodegradable mesh stimulates collagen production and angiogenesis:  
an approach to improving biomaterial integration in pelvic floor repair**

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## **Background**

Polypropylene meshes cause severe complications in some patients. Materials that are biomechanically compatible and can better integrate into host tissues are urgently needed.

## **Objective**

To design an estradiol-releasing electrospun poly-L-lactic acid (PLA) mesh and evaluate its ability to stimulate new extracellular matrix and new blood vessel formation.

## **Design, setting and participants**

Human adipose derived mesenchymal cells (ADMSC) were isolated from fat. PLA meshes with micro to nano sized fibres containing 1%, 5% and 10% estradiol were constructed and used for *in vitro* and *in vivo* experiments.

## **Interventions**

The angiogenic potential of the fibrous meshes was evaluated using an *in vivo* chorioallantoic membrane (CAM) and an *in vitro* chick aortic arch assays.

## **Measurements**

Estradiol release was measured fluorimetrically. The effect of fibrous meshes on proliferation and extracellular matrix (ECM) production of ADMSC was assessed by immunohistology. Mechanical properties were tested using a tensiometer.

## **Results and limitations**

The ultrastructure of the mesh was not affected by the inclusion of estradiol and mechanical properties were only slightly modified. Estradiol was released from PLA meshes over a 5-month period. ADMSCs cultured on estradiol-releasing PLA meshes produced more ECM involving collagen I, III and elastin. Estradiol-releasing meshes doubled new blood vessel formation in the CAM assay ( $p= 0.001$ ) and outgrowth of pro-angiogenic cells in the aortic arch assay ( $p= 0.001$ ). Further studies in longer term animal models are required to confirm these results.

## Conclusion

Estradiol releasing PLA meshes increase ECM production and stimulate angiogenesis. As such, they are promising candidate materials to be used in pelvic floor repair and to improve the initial healing phase of a repair material following implantation.

## Patient summary

In this study we designed a tissue engineered material to be used to support weakened pelvic floor tissues in women to avoid the complications associated with current surgical mesh. Our results showed that this material can stimulate new blood vessel formation in simple chick assays and tissue production *in vitro*. Both properties should help in integration of this material into patients tissues and merit further study in physiologically relevant animal models.



## 1. Introduction

In the 1990s, Petros *et al* reported the implantation of PPL mesh tapes to treat stress urinary incontinence (SUI) <sup>1</sup>. Following this, larger areas of mesh were used to repair pelvic organ prolapse (POP), however, serious complications have now been demonstrated in up to 15% of women <sup>2</sup> and to a lesser degree in SUI surgery <sup>3</sup>. The US Food and Drug Administration released two public health warnings related to mesh complications in 2008 and 2011 <sup>4</sup> and in 2016 re-classified trans-vaginal mesh devices from class II to III <sup>5</sup>. Subsequently these led to a reduction in the number of trans-vaginal mesh surgeries performed for POP from 27% to 2% <sup>6</sup>, with a more modest decline in the number of mid-urethral tapes inserted to treat SUI. Instead, there appears to be an increase in the number of no- mesh surgeries such as autologous fascia sling operations <sup>7</sup>.

PPL devices evolved over many years from an initial metal wire mesh <sup>8</sup> to the current monofilament, macroporous mesh <sup>9</sup>. PPL mesh was not designed nor evaluated for safety in the pelvic floor prior to its introduction. The mechanical properties, microbial flora and vascularization status of paravaginal tissues are very different to that of the abdomen. Recent data has demonstrated site-specific responses to implanted PPL mesh; contraction and exposure of the mesh occurred following trans-vaginal implantation in sheep, while no complications were demonstrated following abdominal implantation over 12 months <sup>10</sup>. Furthermore, PPL mesh undergoes an M1 (proinflammatory) macrophage response, characterized by expression of pro-inflammatory cytokines, which leads to a vigorous foreign body reaction <sup>11</sup>.

Materials that are specifically designed for implantation into the pelvic floor are urgently required <sup>12</sup>. In exploring several candidate materials, we identified electrospun poly-L-lactic acid (PLA) mesh as a material, which possess mechanical properties more closely related to those of healthy paravaginal tissues <sup>13</sup> and it induces an M2 macrophage response associated with constructive remodelling <sup>11</sup>. Electrospun PLA may therefore lead to better integration during the initial healing phase following implantation, whilst providing appropriate structural support for tissues.

A well vascularized wound bed is crucial to achieve rapid integration of materials following implantation. Clinical studies report a decrease in vaginal blood flow in postmenopausal

women, which improves significantly after estrogen replacement <sup>14</sup>. 17- $\beta$  estradiol plays a vital role in maintenance of normal structure and function of pelvic tissues. It is an inhibitor of matrix metalloproteinases <sup>15</sup> and stimulates neo-collagen synthesis <sup>16</sup> and directly stimulates vascular endothelial cells through the estrogen receptor <sup>17</sup>.

A potential solution to improving the angiogenic potential of repair devices is to incorporate pro-angiogenic drugs into materials <sup>18</sup>. In this respect estradiol is an attractive agent as it has a known safety profile and is in clinical use for this purpose. The aim of this study was to develop an estradiol-releasing electrospun mesh of PLA, evaluating its ability to support new tissue formation and to stimulate neovascularization to improve the integration of materials into the wound bed for urogynaecological applications.

## **2. Materials and Methods**

### ***2.1. Electrospinning***

Poly-L-lactic acid (Goodfellow, Cambridge, UK) at 10% (wt/v) was dissolved in dichloromethane (DCM). 17- $\beta$  estradiol (Sigma-Aldrich) was dissolved in DCM added at 1%, 5% and 10% by mass of polymer to produce colourless solutions. 20mls of polymer solutions were loaded into syringes fitted with 21G needles, placed into a syringe pump (Genie<sup>TM</sup>Plus, Kent Scientific, USA), and delivered at 40 $\mu$ l/min per syringe. Random microfibres were created at an accelerating voltage of 17kV DC and collected on an aluminium foil covered earthed mandrel rotating at 300rpm.

### ***2.2. Scanning Electron Microscopy (SEM)***

Samples were mounted on 12.5mm stubs and sputter coated with 25nm of gold (Edwards sputter coater S150B, Crawley, England). SEM (Philips/FEI XL-20 SEM; Cambridge, UK) was performed at an accelerating voltage of between 10-15 kV and a SPOT size between 2 and 3. Fibre diameter and pore size were measured using ImageJ software (National Institute of Health)<sup>19</sup>.

### ***2.3. Release of estradiol from the fibrous meshes***

Meshes were cut to 15mm<sup>2</sup>, weighed and incubated in 1ml/well of Phosphate buffered saline (PBS). The estradiol concentration released from each of the 3 mesh groups (10mg (1% wt/vol), 50mg (5% wt/vol) and 100mg (10% wt/vol)) over 5 months was measured fluorimetrically (Kontron SFM 25 spectrofluorimeter) at  $\lambda_{ex}$ 277nm/ $\lambda_{em}$ 310nm against solutions of known concentration (n=6 per group). Fresh PBS was replaced after each sample was removed.

### ***2.4. Culture of Adipose-derived mesenchymal stem cells (ADMSC) on meshes***

ADMSC were cultured from human subcutaneous fat as described previously<sup>13</sup>, donated under an HTA research tissue bank license (08/H1308/39). Cells were cultured in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Advanced Protein Products, Brierley Hill, UK), 2mM glutamine, 0.625 $\mu$ g/mL amphotericin B, 100IU/mL penicillin and 100 $\mu$ g/mL streptomycin (Gibco Invitrogen, Paisley, UK).

Meshes were cut to 15mm<sup>2</sup> and sterilized in 70% ethanol. 500,000 passage 3 ADMSC were

seeded per mesh and samples cultured for 2 weeks at 37°C, 5% CO<sub>2</sub>. The metabolic activity of seeded cells was quantified by AlamarBlue™ (Sigma-Aldrich, Dorset, UK) assay at days 7 and 14, as described previously<sup>19</sup>.

### ***2.5. Assessment of ability of ADMSC to proliferate and produce extracellular matrix on estradiol releasing fibrous meshes***

Samples were fixed with paraformaldehyde after 2 weeks of culture and stained with 1ml of 1ng/ml of 4',6-diamidino-2-phenylindole dihydrochloride ((DAPI) Gibco Invitrogen, Paisley, UK). After incubating with bovine serum antigen (BSA), following primary antibodies were added (all AbD Serotec, Oxford, UK) and incubated for 1 hour: 100µl of 20µg/ml each of goat anti-human collagen I and collagen III and 100µl of 0.25mg/ml rabbit anti-human alpha elastin at concentrations of 20µg/ml, 20µg/ml and 0.25mg/ml, respectively in BSA.

Secondary antibodies were subsequently added and included 100µl of 20µg/ml FITC labelled rabbit anti-goat IgG for collagen I/III and 100µl of 20µg/ml FITC labelled goat anti-rabbit IgG for elastin and incubated for 1 hour.

DAPI stained nuclei and FITC labelled matrix components were imaged using a fluorescent microscope (Axon ImageXpress™, Molecular Devices Limited, Union City, CA) by switching between filters for DAPI ( $\lambda_{ex}$ 385nm/ $\lambda_{em}$ 461nm) and FITC ( $\lambda_{ex}$ 495nm/ $\lambda_{em}$ 515nm).

Total collagen production was measured by Sirius Red staining as described previously using a 0.1% solution of Sirius Red F3B in saturated picric acid<sup>19</sup>.

### ***2.6. Assessment of effects of estradiol on differentiation capacity of ADMSC***

ADMSCs at passage 3 were cultured with 15mm<sup>2</sup>, weight matched sections of either estradiol-releasing or control PLA meshes in DMEM (n=6). After 24 hours, media was replaced with specific induction medium and cultured for 3 weeks. Adipogenic and osteogenic induction media contained the following supplements in addition to above defined DMEM: 1 µM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 µg/mL insulin and 100 µM indomethacin for adipogenic media and 10 nM dexamethasone, 50 µg/mL ascorbate-2-phosphate and 2 mM β-glycerophosphate for osteogenic media.

Cells were fixed and stained with Oil Red O and Alizarin Red for adipogenic and osteogenic phenotypes, respectively. Counter staining was performed using haematoxylin. Histological

differences were assessed blindly by 6 post-doctoral researchers. All reagents were from Sigma-Aldrich, Dorset, UK.

### ***2.7. Assessment of effect of estradiol on mechanical properties of fibrous meshes***

Meshes were cut into 5mm x 10mm sections and clamped into a tensiometer (BOSE Electroforce Test Instruments, MN) using a 22N load cell and a ramp test at a rate of 0.1mm/sec. Results were standardized by width and thickness of materials (N/mm<sup>2</sup>). Tests were performed both before and after 7 days of ADMSC culture on the meshes.

The initial linear gradient of each resulting stress vs strain curve was taken as the Young's modulus (N/mm<sup>2</sup>), while the ultimate tensile strength (UTS) was taken as the force per area of sample at break/maximal point. Both values were compared to data published for paravaginal tissues of healthy premenopausal patients <sup>20</sup>.

### ***2.8. Assessment of angiogenic potential of estrogen releasing meshes***

Angiogenesis was assessed using an *ex ovo* chick chorionic allantoic membrane (CAM) and an *in vitro* chick aortic arch assay. Pathogen-free fertilized white leghorn chicken eggs (*Gallus gallus domesticus*) were obtained from Henry Stewart Co. (UK). Care was consistent with the Home Office, UK guidelines. Embryos were cultured using an *ex ovo* (shell less) method <sup>21</sup>. Embryos were transferred into a petri dish on embryonic development day (EDD) 3 and maintained in a humidified incubator at 38°C between EDDs 3 to 14. Embryo survival was assessed daily.

#### **The *ex ovo* CAM assay**

Meshes were cut into 6.5mm diameter circles using an Epilog Laser Cutter (Clevedon, UK) and sterilized under UV light. At EDD 8, two meshes were placed on the CAM surface. At EDD 14, images of meshes and surrounding CAM area were taken with a digital microscope. After imaging, 0.1mL of a 20% solution of Lens culinaris agglutinin [LCA (Vector Laboratories)] was injected into the circulation using a 30G hypodermic needle to fluorescently label intraluminal endothelial cells. Embryos were sacrificed and meshes were removed from the CAM surface. Angiogenesis was quantified by counting all discernible blood vessels traversing a 1mm annulus about the 2mm imaginary circle drawn around the mesh <sup>22</sup>.

### Chick aortic assay

Embryos were sacrificed on EDD 14, the aortic arch removed and cut into 1mm rings under a stereomicroscope. Aortic rings were embedded in 50µL of Matrigel® (Basement Membrane Matrix, Corning®) and cultured in Minimum Essentials Media supplemented with 2.5% FCS, 50 units/mL penicillin and 50µg/mL streptomycin (GIBCO, Carlsbad, CA). Rings were co-cultured with meshes using an insert (Greiner Bio-One GmbH). Endothelial sprout length was observed under an inverted microscope from day 5. Endothelial cells were characterized by immunofluorescence staining with 10 µg/ ml Isolectin B4 (Vector Laboratories, Burlingame, USA).

### Histology

Meshes and surrounding CAM were fixed with paraformaldehyde, and embedded in freezing medium. 6µM sections were taken using a cryostat (Leica CM1860UV, Leica, Germany). Slides were stained with DAPI and observed under an epifluorescence microscope (Olympus, Japan).

### **2.9. Statistics**

Statistical differences between two group means were analysed using Student's T test when the data was normally distributed and with Mann Whitney U test otherwise. Survival of the chick embryos was analysed with the Kaplan Meier method. A p value of <0.05 was considered statistically significant.

### 3. Results

#### **3.1. Effect of estradiol on mesh ultrastructure**

There was no significant difference between the fibre diameter and pore size of PLA versus estradiol-releasing PLA meshes ( $0.65\pm 0.30\ \mu\text{m}$  vs  $0.71\pm 0.28\ \mu\text{m}$  [ $p=0.47$ ] and  $4.26\pm 2.29\ \mu\text{m}$  vs  $4.25\pm 2.04\ \mu\text{m}$  [ $p=0.98$ ], respectively) as shown in Figure 1. The lipophilic estradiol could effectively be loaded directly into the fibres of the PLA as demonstrated by the absence of visible drug crystals on the fibres' surface.

#### **3.2. Release of estradiol from meshes over 5 months**

The cumulative release of estradiol increased for each time-point, until no further estradiol was released as demonstrated in Figure 2. A burst release of between 40- 50% of the drug was observed in the first 14 days. The cumulative release of estradiol was roughly proportional to the concentration of estradiol loaded-the percentage release over 133 days was of the order of 1.5 to 2.5% -calculated to be 2.5%, 1.4% and 2.45% of the initial estradiol present in the polymer solution for the 10mg, 50mg and 100mg estradiol meshes, respectively.

#### **3.3 Effect of estradiol releasing meshes on differentiation capacity of ADMSC**

Culturing ADMSC in specific induction media resulted in cellular differentiation over 3 weeks, including the formation of lipid vesicles for ADMSC cultured in adipogenic medium and the presence of calcium for cells cultured in osteogenic medium (Figure 3). There were no significant differences in cell morphology, lipid vesicles or calcium content between control and estradiol-releasing meshes, as assessed using blind scoring.

#### **3.4 Effect of estradiol on metabolic activity and extracellular matrix production of ADMSCs**

Figure 4A demonstrates an increase in metabolic activity from 7 to 14 days for cells cultured on both estradiol and control meshes, with a greater increase seen for cells cultured on estradiol meshes ( $p<0.01$ ). ADMSCs cultured on estradiol meshes for 14 days produced a greater proportion of total collagen than cells cultured on control meshes ( $p<0.001$ ) as demonstrated in Figure 4B. Figure 4C shows a greater proportion of collagen I and III and elastin from culture of ADMSC on estradiol meshes compared to controls, a finding which reaches statistical significance for collagen III and elastin as demonstrated in table 1.

**Table 1.** Quantification of extracellular matrix component expression using a blind scoring system. Mean  $\pm$  standard deviation (SD) of 5 representative immunohistochemistry images for each of collagen I, collagen III and elastin as assessed by 6 blinded post-doctoral reviewers. Scoring system includes 0 – absence, 1 – mild presence, 2 – moderate presence, 3 – large presence, 4 – abundance, 5 – great abundance.

	Collagen I		Collagen III		Elastin	
	Estradiol	No estradiol	Estradiol	No estradiol	Estradiol	No estradiol
<b>Mean</b>	4.0	3.50	2.67	1.17	2.83	1.33
<b>SD</b>	0.63	0.55	0.52	0.41	0.52	0.41
<b>P value</b>	0.1739		0.0002		0.0002	

### ***3.5 Effect of estradiol on the mechanical properties of meshes***

Figure 5A demonstrates that estradiol-releasing meshes show a Young's Modulus closer to the values of healthy native fascia. After 2 weeks of cell culture, both meshes become more elastic, however estradiol-releasing meshes were significantly stiffer ( $p < 0.01$ ) than control meshes. Figure 5B demonstrates that estradiol-releasing meshes are significantly stronger than controls, both before and after 2 weeks of cell culture. Of note, the UTS of control meshes decreases after 14 days of cell culture, while the UTS of estradiol-releasing meshes increases.

### ***3.6 Effect of estradiol on the angiogenic properties of PLA meshes***

The CAM assay demonstrates at least double the number of blood vessels growing towards the estradiol meshes compared to controls over 7 days (mean vessel count: 25.0 ( $\pm 5.29$ ) and 10.67 ( $\pm 2.64$ ) respectively,  $p < 0.001$  as shown in Figure 6A and B). Estradiol did not affect embryo survival rates (Figure 6C).

Estradiol-releasing PLA meshes induced longer and denser endothelial sprouts when co-cultured with chick aortic rings (Figure 7A). Figure 7C demonstrates a sprout length/radius of 1.6 ( $\pm 0.51$ ) and 1.0 ( $\pm 0.44$ ) for estradiol-releasing and control meshes respectively ( $p = 0.01$ ) (Figure 7C).



Host tissues infiltrated into each mesh to a similar degree (Figure 8). There was an increased number of blood vessels (stained with fluorescent angiography) in the CAM tissue adjacent to and in- between the fibres of the estradiol-releasing meshes compared to controls. After incubation in the *in vivo* environment for 7 days, meshes contracted to a size of 43.2% ( $\pm 1.1$ ) and 41.2% ( $\pm 8.3$ ) of their original area for estradiol and control meshes, respectively ( $p=0.48$ ).

## 4 Discussion

Ideally biodegradable repair materials should integrate well into the patient's tissues and be strong enough to provide structural support, while regeneration of host tissues over time eventually assumes this load bearing function.

In this study we demonstrate that incorporation of estradiol into the fibres of an electrospun PLA mesh do not significantly alter the overall microstructure of the mesh. The estradiol releasing PLA mesh was more elastic and stronger compared to control PLA meshes after culturing with cells *in vitro* for 14 days. This can be attributed to the stimulatory effect of estradiol on cells and/ or direct plasticizing effect of estradiol, as there is a difference in Young's modulus and ultimate tensile strength before culturing. Tissue integration was assessed in this study through the interaction of cells *in vitro* with the mesh and the ability of the mesh to stimulate angiogenesis using *in vivo* and *in vitro* models. For the former, ADMSC were selected as a representative cell source, capable of undertaking a range of phenotypes. Estradiol was released over a five-month period in a dose-dependent manner. Meshes consisted of random micro/ nano-fibres with a range of pore sizes up to 10µm. While porosity influences the ease of cell infiltration into the mesh, our previous studies with electrospun PLA implanted in rats<sup>23</sup> and rabbits<sup>11</sup> demonstrate a rapid integration of host tissues into these with an M2 macrophage (tissue remodelling) response. Several assessments of non-degradable polypropylene meshes in dogs<sup>24</sup>, sheep<sup>10</sup>, rhesus macaque<sup>25</sup> and humans<sup>26</sup> demonstrate sustained chronic inflammation. Biodegradable PLA meshes last intact for at least one year when implanted in rat models<sup>23</sup>. In the current study we demonstrate that inclusion of estradiol improved the ability of ADMSCs to stimulate new ECM production *in vitro* including collagen I, III and elastin.

The most critical feature of this estradiol-releasing PLA mesh is the significant increase seen in the number and density of blood vessels and integration of tissues into this material when implanted into the CAM assay. The CAM assay is an established method for the *in vivo* evaluation of angiogenesis, which allows direct visualization of the newly forming vessels into a biomaterial<sup>27</sup>.

An estradiol releasing electrospun mesh has previously been described for use as a wound care product in post-menopausal women, where the anti-inflammatory properties of

estradiol were utilized<sup>28</sup>. Estradiol has been demonstrated to stimulate endothelial cells to produce VEGF when released from a collagen based mesh<sup>29</sup>. Here we demonstrate for the first time that estradiol released from an electrospun mesh dramatically stimulates new blood vessel formation in and around the material. Although we have designed this material for use in pelvic floor repair, it could have wider applications in all areas of tissue engineering as a pro-angiogenic mesh.

The promising results obtained in this study will now need to be confirmed in physiologically relevant animal models to evaluate the potential for progressing to the clinic. Animal studies will particularly be helpful to evaluate the inflammatory response to estradiol releasing PLA meshes as the chick embryo used in this study does not have a mature immune system. Also the size of the mesh material used in our experiments are much smaller than that would be required in clinical practice and materials need to be implanted under load for many months to assess their mechanical properties and longer term tissue integration.

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## Reviewer 1

We thank the reviewer for evaluating our work.

## Reviewer 2

1. this study lacks the final experiment of surgical implantation in mammals of PLA meshes loaded with oestrogen and ADSCs. This is particularly important because ADSCs may exert protection on lesioned tissues, potentiate differentiation of resident stem cells or even differentiate in other cell types, all of which could impact on the beneficial effects on blood vessel formation.

### Our reply:

We thank the reviewer for this comment. The need to confirm these promising results in an animal model has now been added to the discussion as a limitation of this study. In this study the ADSCs were used to assess the ability of the scaffolds to allow cell penetration as we hope would occur in vivo.. Thus we do not, in this study propose implantation of our scaffolds with ADSCs on them.

It is a possibility for the future and we have just started to look at this possibility but recognise that it will take significant work and will be a bigger regulatory hurdle to overcome than implanting a membrane releasing a known pro-angiogenic hormone already in clinical use.

The word limit of the manuscript prevents us going into this anything other than a cursory fashion which we now do in the discussion.

2. I suggest reviewing the writing of the manuscript. At some points, it still reads more like a draft than the final version. For example, the dilution of the primary antibodies against collagen and elastin are not referred nor if the intensity of immunolabelling of elastin was evaluated. Also, the legends could give a bit more detail.

### Our reply:

We thank the reviewer for this comment and the manuscript has now been revised and further detail particularly in the legends have been added. The dilution of primary antibodies are also added together with more detail on intensity of immunolabelling of collagen I, III and elastin.

3. Lastly, I assume red arrows are pointing to blood vessels as it not indicated in the legend nor the magnification allows the identification of capillaries, arterioles or venules.

### Our reply:

We apologise for this. The legend is now modified accordingly. The main point of 40X H&E images was to demonstrate the ability of the scaffolds to allow ingrowth of small blood vessels in-between the fibres of the scaffolds. Higher magnifications make it very hard to focus on a single plane to demonstrate either the blood vessels or the PLA fibres, which does not show our point. That is why we need this image as it is but we have added detail to the legend.

### Reviewer 3

1. they do not describe in M&M how Estradiol is bound/incubated into mesh, which is I think an essential step to include (I do not know if this is because of patents).

Our reply:

We thank the reviewer for this comment. This information is now added to the text in the results section. Briefly, since estradiol it is a highly lipophilic steroid and it dissolves well in the polymer solution, it can be loaded effectively into the fibres of the electrospun PLA. In other words we have electrospun a drug/ polymer blend.

2. Mesh is not investigated in an animal model, which I consider a limitation of this study.

Our reply:

We agree with the reviewer. This point is added to the discussion as a limitation of the study and something that is the next logical step to achieve.

3. 'It appears that we are now back to a pre- mesh era with some important lessons to be learned.' This is a very incorrect statement. There is a lot of room for improvement, but the amount of sling surgery performed is standard care for SUI. We are perhaps more careful who we select for mesh surgery. On the other hand you could state that tissue engineering has so far not produced any widely accepted, commercially available alternative.

Our reply:

We thank the reviewer for this comment. This point has been revised in the text.

4. M&M \_\_\_\_ Please include how Estradiol is incorporated into/onto the mesh.

Our reply:

We thank the reviewer for this comment. This information is now added to the manuscript . Essentially Estradiol is loaded directly into the fibres of the PLA as we spin it. This is possible because of the lipophilic nature of this steroid so it can go into an organic solvent readily.

5. There appears to be considerable contraction of the mesh in figure 6. Has diameter of scaffolds decreased over time?

Our reply:

We thank the reviewer for this careful observation. Yes, the scaffolds contracted to nearly 40% of their original surface area as the scaffold was infiltrated with new tissue over 7 days *in vivo*. This point is now added to section 3.6 with exact figures.

6. Can authors explain the different release % between Estradiol meshes (the 50 mg mesh is quite a bit lower than the others).

Our reply:

The release of Estradiol from electrospun PLA scaffolds is both diffusion and degradation dependent. In other words, we see a burst release of Estradiol due to the presence of non-incorporated drugs in the surface together with a more sustained/ gradual release of small amounts of Estradiol over a longer period of time as the scaffolds degrade.

The rate of release was broadly proportional to the amount of drug present in the scaffold. The small difference in percentage release calculated for the 50 mg scaffold we suggest is not significant in the overall scheme of things - for the 3 scaffolds the range of release was from 1.4% to 2.5%- we have altered the text slightly to indicate the range of release – we do not want to put undue emphasis on there being less release from the 50 mg compared to other scaffolds. Figure 2 shows that the cumulative release over time was much as one would expect it taking into account the amount of estradiol loaded into each scaffold.

7. Figure 5: Can Authors explain how Estradiol appears to influence tensile strength before cell culturing (Young's Modulus ) compared to control??

Our reply:

The reviewer has rightly raised this point. The only possible explanation we can suggest is that estradiol might be acting as a plasticizer when incorporated into the fibres of the electrospun PLA which could then change the Young's modulus and ultimate tensile strength of the scaffolds. This point is now mentioned in the discussion.

8. Also I would like a comment in the discussion concerning the quite drastic decrease in N/mm<sup>2</sup> after 14 days of culturing (below normal fascia values). Does this further decrease after 14d? This has potential severe implications on the applicability of this mesh in patients (you need mesh that gives support for much longer periods than 14d). There is no mention of any of this potential severe limitation in the discussion.

Our reply:

We thank the reviewer for this important comment. The ultimate tensile strength of PLA scaffolds decreased significantly 14 days after culturing them with ADSCs *in vitro*. The main point we wanted to demonstrate was that presence of estradiol within the PLA scaffolds prevented this unwanted effect on the mechanical properties *in vitro*. This could be due to the production of new extracellular matrix produced by ADSCs that might have replaced some of the functionality of the ECM and/ or it might be due to the plasticizing effect of estradiol on scaffolds.

For PLA only scaffolds, the reviewer is right to think that the ultimate tensile strength might further degrade after 14 days with cells -however this 2 week snapshot is only part of the picture. As we acknowledge in the discussion the next logical step for this work is to progress it into an *in vivo* model where the scaffolds need to be implanted for long enough ( probably 3 months as we have previously done in rabbit experiments working with colleagues in Leuven ) to be able to comment sensibly on the mechanical properties of the scaffolds after several months of implantation. Even this is not ideal- we really need to be able to implant scaffolds for at least a year in a large animal model such as a sheep. As the reviewers will be aware this is not a trivial experiment but the very promising results we have found from this *in vitro* study now suggest that we should seek the funding and resources to take this material into a larger animal experiment and run it for a much longer time.



When doing such animal experiments the scaffolds would be implanted without cells and the impact of including oestradiol on the tissue integration of the scaffolds and their mechanical properties would be assessed.

9. Resolution of images figure 7 B is poor and could use a higher magnification to clearly show content. Also images for figure 3 and 7B are too dark to interpret

Our reply:

We have now corrected the brightness of the figures 3 & 7 and changed figure 7B.

10. Discussion.

There is no critical discussion on limitations of the study.: examples: 1) The further need to investigate the mesh in an animal model. 2) That an animal model is perhaps better to investigate inflammatory responses. 3) That only small mesh sizes has been investigated and not larger ones that would be needed for clinical use. 4) The use of ADMSC 5) what are the current prospects for using these meshes in the clinical studies, have they been done /how does this model relate to these...

Our reply:

We thank the reviewer for this comment. A paragraph about the limitations of our study and where it needs to go next to be able to comment on tissue integration and mechanical properties has now been added to the discussion.

Figures

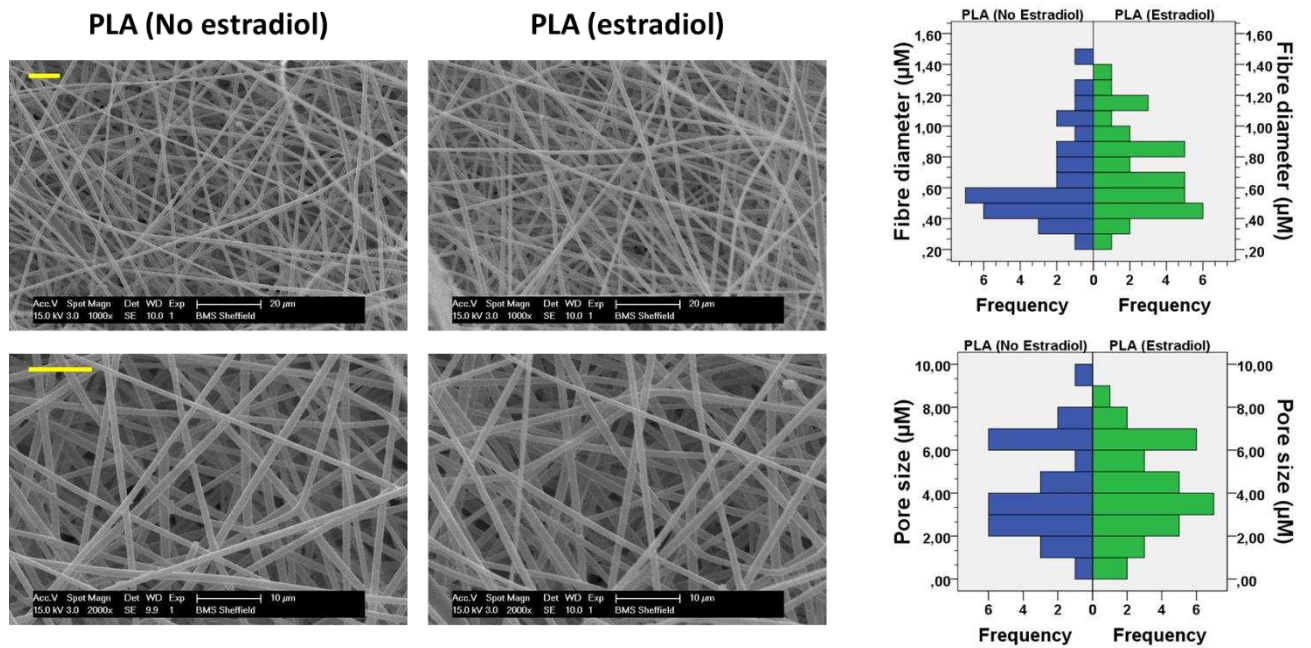


Figure 1. Evaluation of the microstructure of the electrospun PLA only meshes and estradiol releasing PLA meshes by scanning electron microscopy. Micro- nano sized random fibres and the microporous structure of the mesh can be observed. Histograms on the right demonstrate distribution of fibre diameters and pore sizes in both meshes. Scale bars represent 10 µm.

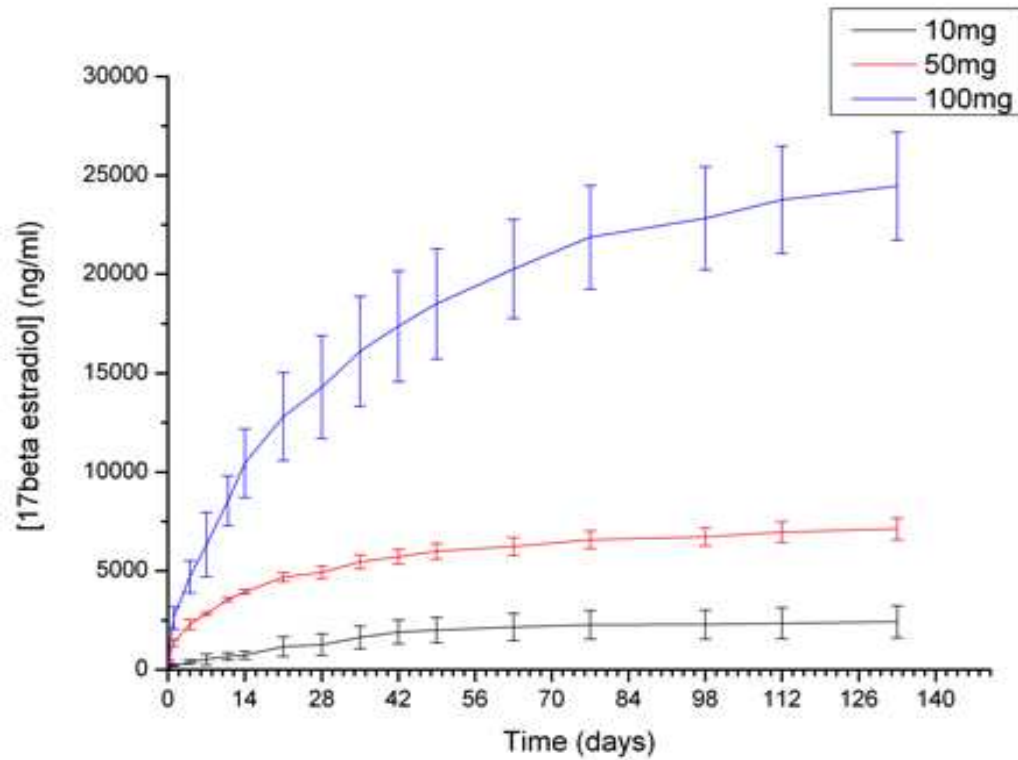


Figure 2. Cumulative release of estradiol from 3 different estradiol releasing meshes over 133 days in a concentration dependent manner. Estradiol is gradually released from meshes after an initial burst release in the first 14 days. Measurements done using a spectrofluorometer ( $n=6\pm SEM$ ).

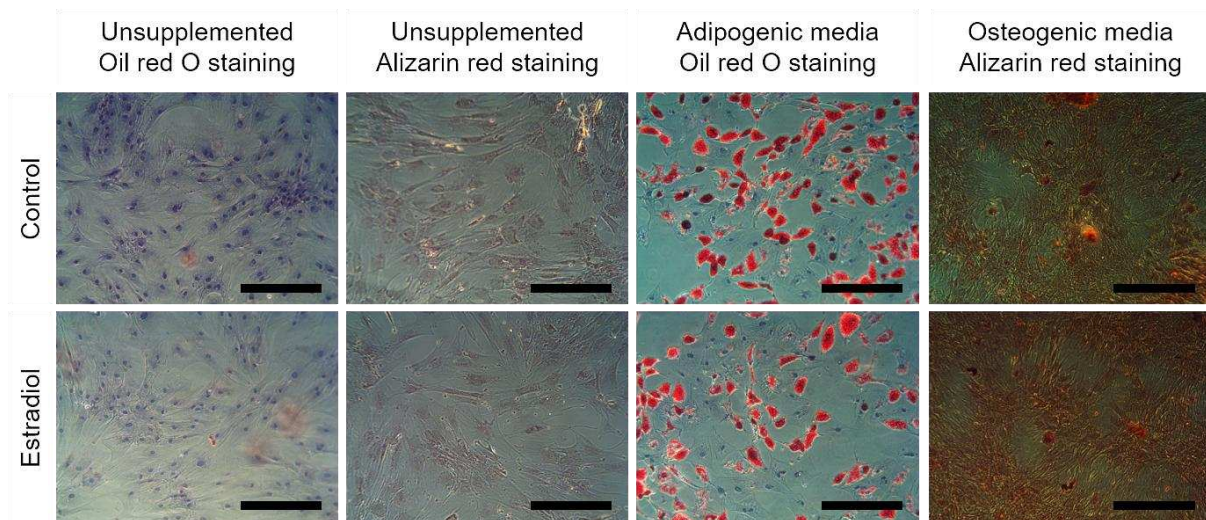
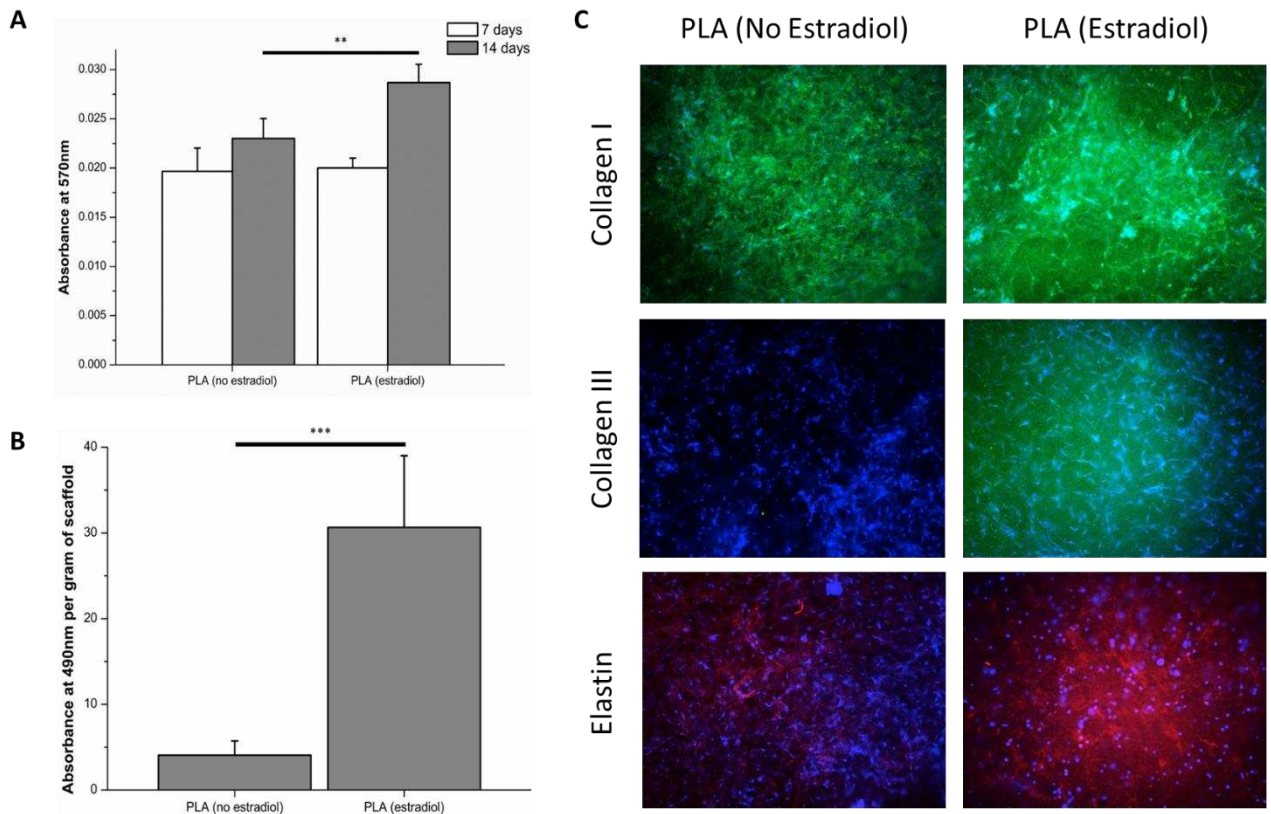


Figure 3. Effect of estradiol releasing meshes on the differentiation capacity of adipose derived mesenchymal stem cells (ADMSC). ADMSCs cultured either in DMEM, adipogenic media or osteogenic media, stained with haematoxylin and Oil red O (for lipid vesicles) or Alizarin red (for calcium). Scale bar represents 50 $\mu m$ .



**Figure 4.** Extracellular matrix production by ADSC cultured on Estradiol releasing PLA meshes compared to PLA only meshes. (A) Cell metabolic activity of ADSC cultured on control and estradiol releasing PLA meshes as measured by AlamarBlue assay with absorbance measured at 570nm using plate reading colourimeter. ( $n=6 \pm SEM$ ).  $**p<0.01$ . (B) Total collagen production at day 14 as measured by Sirius red assay with absorbance measured at 490nm using plate reading colourimeter. ( $n=6 \pm SEM$ ).  $***p<0.001$ . (C) Fluorescent microscopy images of extracellular matrix components produced by ADSC after 14 days of culture. Collagen I & III (green), elastin (red) and cells (blue). Scale bar represents  $100\mu m$ .

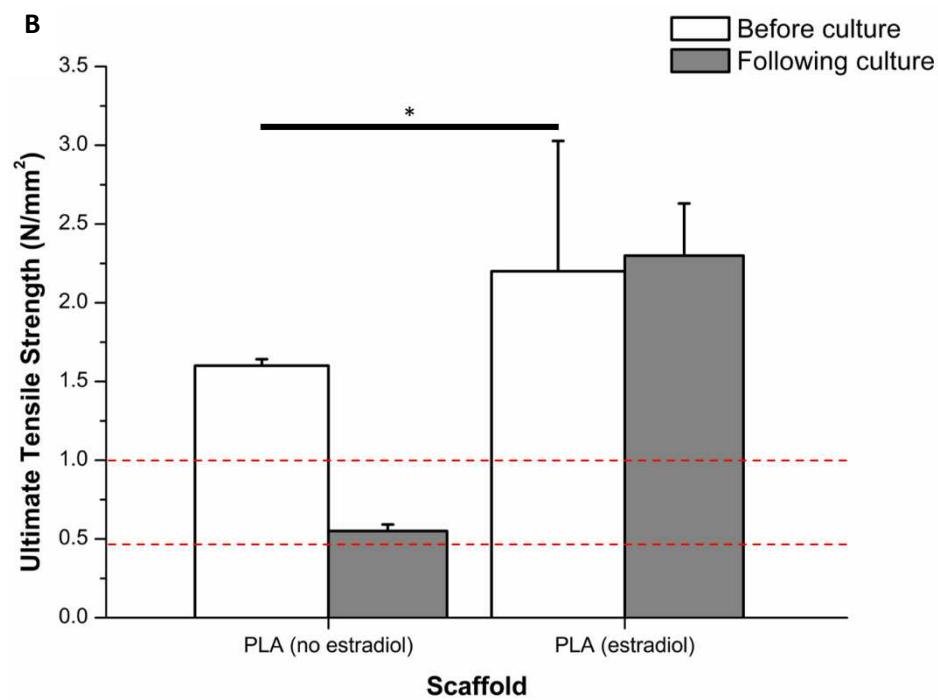
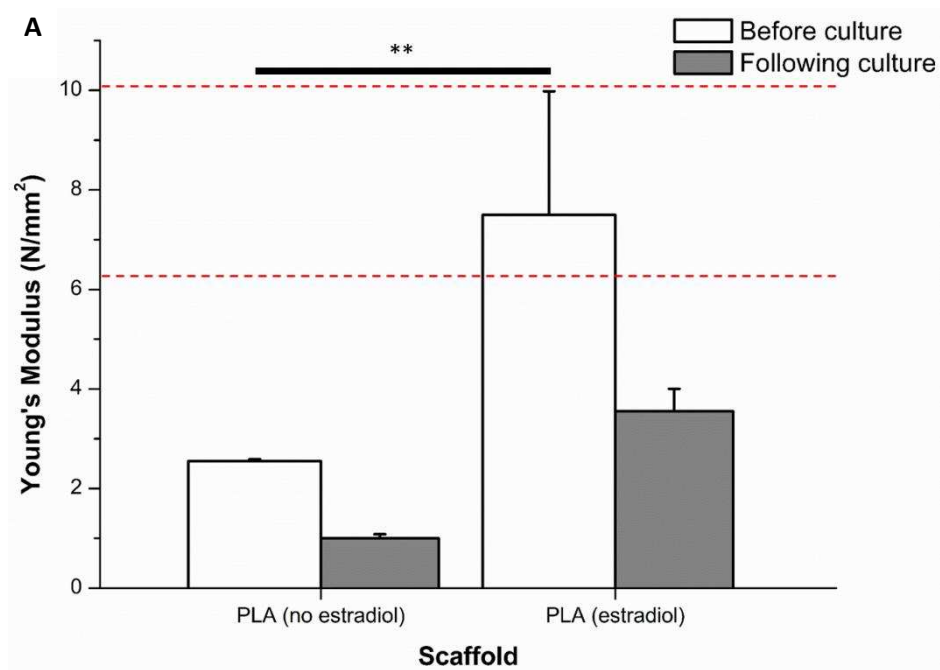


Figure 5. The effect of estradiol on the strength of the meshes assessed in the presence of ADMSCs.

A. Young's Modulus of control and estradiol releasing PLA meshes, before and after 14 days of cell culture with ADSC. Area between dashed lines represents values for healthy native fascia [18].

$N=6\pm SEM$ ,  $**p<0.01$ .

B. Ultimate Tensile Strength of control and estradiol releasing PLA meshes, before and after 14 days of cell culture with ADSC. The area between the dashed lines represents values for healthy native fascia [18].  $N=6\pm SEM$ ,  $***p<0.001$ .

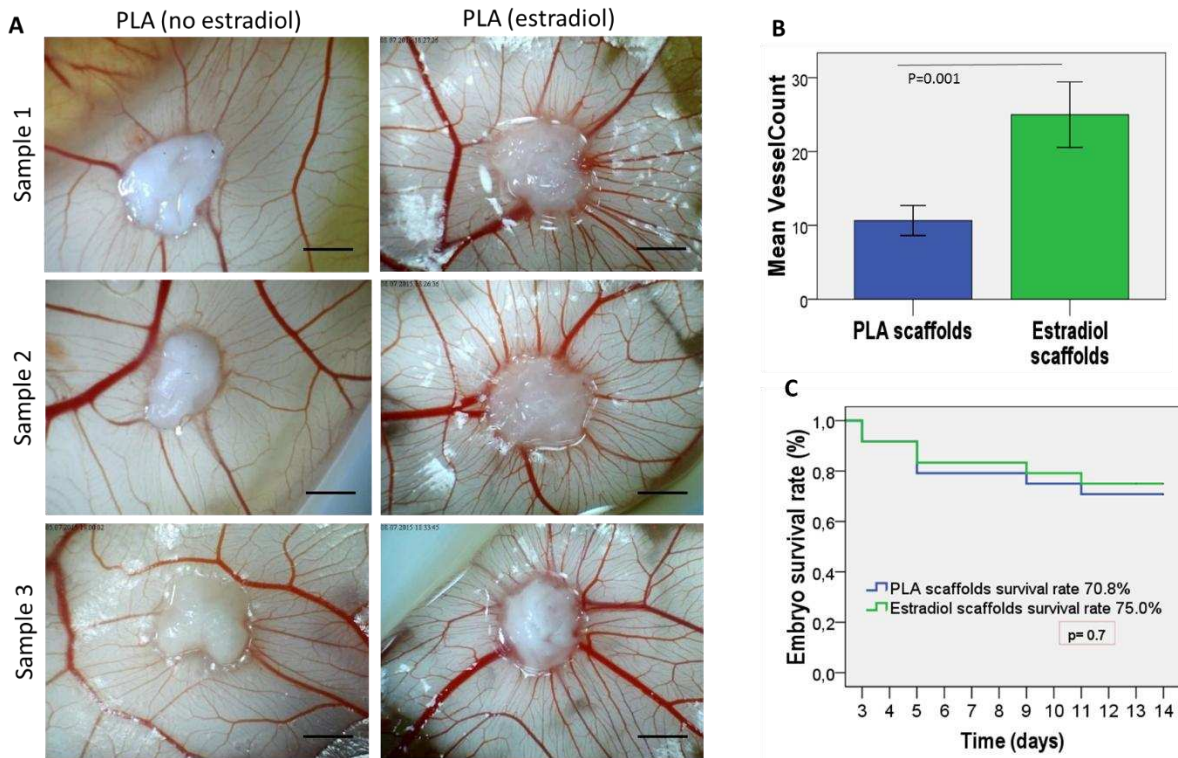
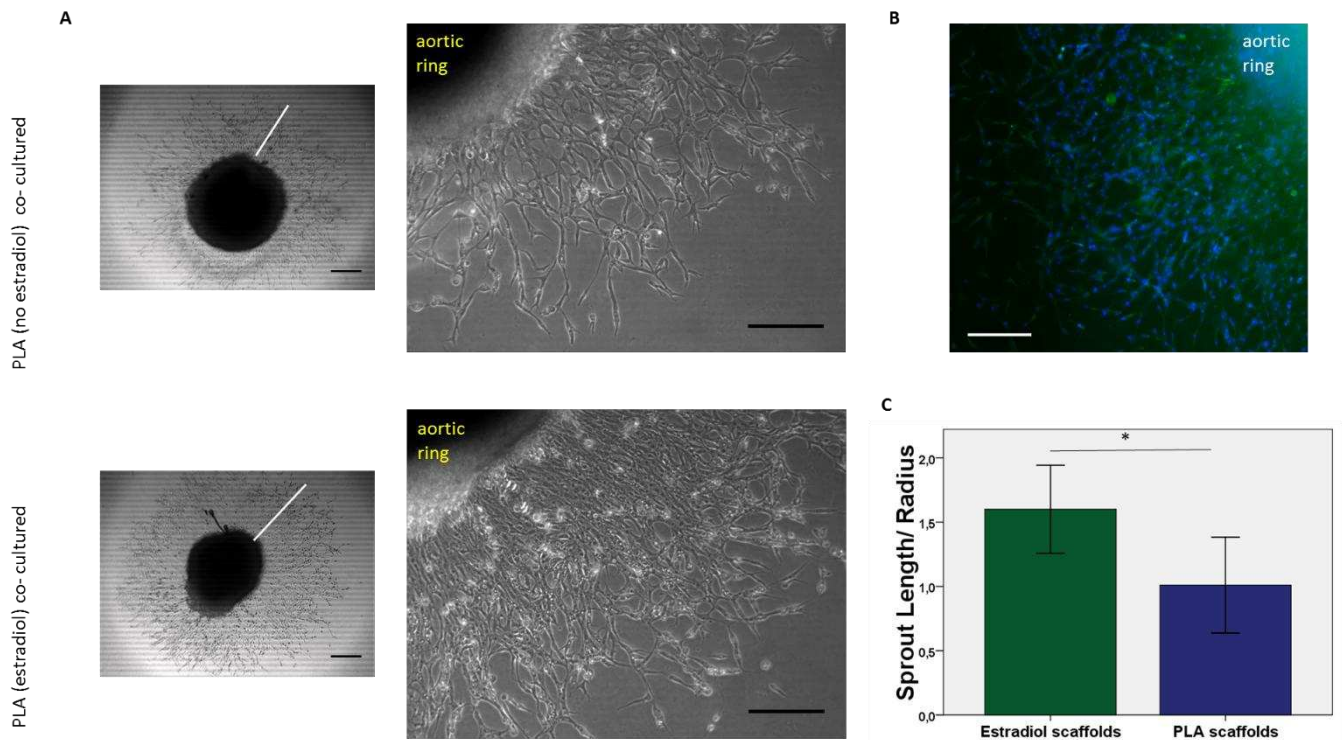
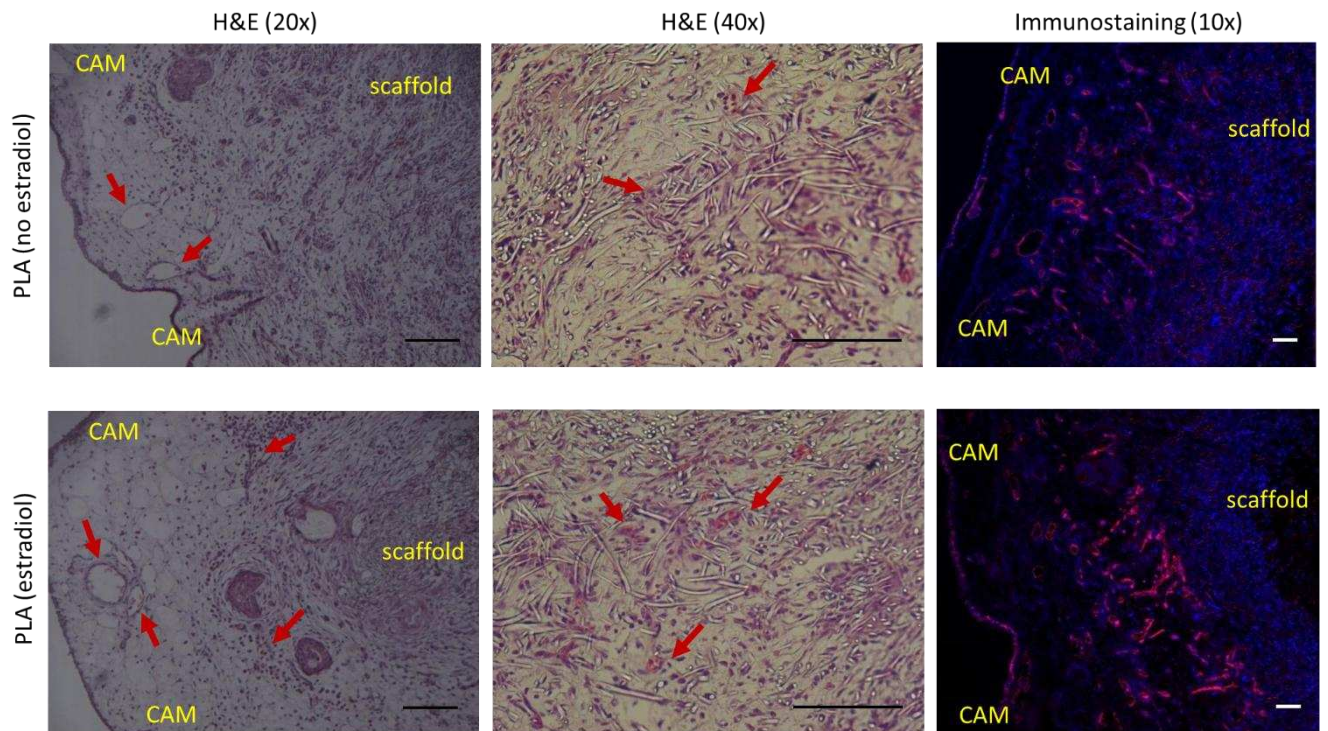


Figure 6. Angiogenic potential of estradiol releasing PLA meshes compared to PLA only meshes, day 14 on chicken chorioallantoic membrane (CAM) assay. A normal distribution of blood vessels was observed when the control PLA mesh was placed on the CAM. With the estradiol releasing mesh newly formed blood vessels can be seen to radiate towards the mesh in a spoke-wheel pattern (A). The mean vessel count of estradiol releasing meshes was double that of the PLA meshes (B) and the embryo survival rate was the same between the two groups (C). (Scale bars represent 3mm).



*Figure 7. A) Evaluation of effect of estradiol on endothelial cell proliferation and sprouting. One millimetre rings from the chick aorta were embedded in Matrigel and co-cultured with PLA only meshes (upper row) or Estradiol releasing PLA meshes (lower row). The extent of endothelial sprouting by 5 days in culture is much greater in the presence of estradiol. B) Fluorescent microscopy images of immunological staining. Lectin IB4 - endothelial cells stained green, nuclear components stained blue (DAPI). C) Sprout length/radius results taken from 11A, \* P=0.01. (Scale bars represent 50  $\mu$ m).*



*Figure 8. Histologic evaluation of tissue- mesh interface. A mild inflammatory reaction together with a normal distribution of blood vessels on the CAM adjacent to the PLA mesh (upper row) can be observed compared to a significantly increased number of large blood vessels in response to Estradiol releasing PLA mesh (lower row) at day 14, Haematoxylen &Eosin (H&E) staining (left side). Also on higher magnification several small blood vessels could be observed in between the PLA fibers which was more abundant in the presence of Estradiol (middle). On the right endothelial cells lining all sizes of blood vessels appear stained Rhodamine- conjugated Lens culinaris agglutinin and cell nuclei are stained with DAPI. Scale bars represent 100  $\mu$ m.*