FGF2 is crucial for the development of bovine luteal endothelial networks *in vitro*

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Abstract

The development of the corpus luteum requires angiogenesis, and involves the complex interplay between factors such as vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2) and platelet-derived growth factor (PDGF). However, the relative role of these factors remains to be elucidated. This study used a new physiologically relevant mixed luteal cell culture system to test the hypotheses that: a) FGF2 and VEGFA are critical for bovine luteal angiogenesis; and b) local luteal PDGF signalling stimulates the formation of endothelial networks. Cells were treated with receptor tyrosine kinase inhibitors against VEGFA (SU1498), FGF2 (SU5402) or PDGF (AG1295) activity. After 9 days in culture, endothelial cells were immunostained for von Willebrand factor (VWF) and quantified by image analysis. Highly organised intricate endothelial networks were formed in the presence of exogenous VEGFA and FGF2. The inhibition of FGF2 activity reduced the total area of VWF staining versus controls (>95%; $P<0.001$). Inhibition of VEGF and PDGF activity reduced the endothelial network formation by more than 60 and 75% respectively ($P<0.05$). Progesterone production increased in all treatments from day 1 to 7 ($P<0.001$), and was unaffected by FGF2 or PDGF receptor kinase inhibition ($P>0.05$), but was reduced by the VEGF receptor inhibitor on days 5 and 7 ($P<0.001$). In conclusion, this study confirmed that VEGF signalling regulates both bovine luteal angiogenesis and progesterone production. However, FGF2 was crucial for luteal endothelial network formation. Also, for the first time, this study showed that local luteal PDGF activity regulates bovine luteal endothelial network formation *in vitro*.

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Introduction

The growth and development of the corpus luteum (CL) requires the establishment of a complex vascular system (Redmer & Reynolds 1996, Reynolds & Redmer 1998). This neovascularisation or angiogenesis is thought to be under the control of several factors, including vascular endothelial growth factor A (VEGFA; Ferrara *et al.* 2003), fibroblast growth factor 2 (FGF2; Presta *et al.* 2005) and platelet-derived growth factor (PDGF; Betsholtz 2004).

VEGFA has been shown to stimulate angiogenesis in the follicle and CL of many species. It is highly expressed in the bovine CL during the early luteal phase (Berisha *et al.* 2000) when extensive angiogenesis is occurring (Zheng *et al.* 1993). VEGFA acts via the tyrosine kinase receptors, VEGFR1 and VEGFR2; with VEGFR2 considered the principal signalling receptor (Ferrara *et al.* 2003). Both receptors have been localised to microvascular endothelial cells of the bovine CL (Gabler *et al.* 2004) with expression shown throughout the luteal phase (Berisha *et al.* 2000). The inhibition of VEGFA action *in vivo* with antibody or antagonist treatment

against VEGFA or VEGFR2 in a number of species results in the profound inhibition of luteal function and angiogenesis, as evidenced by reductions in the number, size and vascularity of corpora lutea and resultant attenuated progesterone secretion (Zimmermann *et al.* 2001, Fraser *et al.* 2005, Yamashita *et al.* 2008).

FGF2 is mitogenic for CL-derived endothelial cells (Gospodarowicz *et al.* 1986) and luteal cells in culture (Grazul-Bilska *et al.* 1995), and stimulated progesterone secretion when infused into bovine corpora lutea (Liebermann *et al.* 1996). In the cow, FGF2 protein was specifically localised to endothelial cells and pericytes of the theca layer prior to the LH surge, but afterwards and in the collapsed follicle, FGF2 protein was found predominantly in the luteinising granulosa cell layer (Berisha *et al.* 2006). However, after a few days, FGF2 was absent from steroidogenic cells but re-appeared in endothelial cells (Berisha *et al.* 2006). We have recently shown that FGF2 protein levels are particularly high during the follicular–luteal transition, suggesting an important role for FGF2 during the development of the

early bovine CL (Robinson *et al.* 2007). Furthermore, injections of FGF2 antibody directly into the bovine CL decreased luteal volume and progesterone production (Yamashita *et al.* 2008).

The PDGF family is composed of four polypeptide chains (PDGFA, PDGFB, PDGFC, PDGFD) which form five dimeric isoforms, with the most common being the homodimers PDGF-AA, PDGF-BB and the PDGF-AB heterodimer. The PDGFs exert their actions via tyrosine kinase receptors; PDGF-AA, PDGF-AB and PDGF-BB act via the PDGF receptor- α (PDGFRA), while PDGF-BB also binds to the PDGF receptor- β (PDGFRB; Fredriksson *et al.* 2004).

PDGF isoforms and both PDGFRs have been localised to the ovary in several species. In the follicle, PDGFs are thought to influence the primordial-to-primary follicle transition (Nilsson *et al.* 2006), to stimulate cell proliferation (Shores & Hunter 2000) and follicle development (Sleer & Taylor 2007a), and influence steroidogenesis (Taylor 2000). In the rat ovary post-hCG, the ligands PDGFA, PDGFB and PDGFC were localised to luteal steroidogenic cells. The PDGFRA was localised to theca-derived luteinising cells of the developing CL, and with further development a sub-population of luteal parenchymal cells and some vascular cells showed immunoreactivity for PDGFRA. By contrast, the PDGFRB was predominantly localised to cells of the luteal microvasculature (Sleer & Taylor 2007b).

Perivascular cells such as pericytes that are closely associated with endothelial cells are important regulators of both the structure and function of vessels (Armulik *et al.* 2005). PDGFB is involved in attracting pericytes to new vessels during angiogenesis via activation of PDGFRB located on pericytes (Betsholtz 2004), and intraovarian PDGF-receptor blockade resulted in a reduction in the number of corpora lutea in the rat and mouse (Sleer & Taylor 2007b, Kuhnert *et al.* 2008). Nevertheless, still very little is known about the role of PDGF in regulating luteal angiogenesis in general and there have been no studies in the cow.

We have recently developed a culture system that incorporates the multiple luteal cell types found *in vivo* (pericytes, fibroblasts, steroidogenic and endothelial cells; Robinson *et al.* 2008). These luteal cells in culture produce increasing concentrations of progesterone over time and respond to LH stimulation. Uniquely, this culture system also mimics luteal angiogenesis *in vitro*; supporting endothelial growth and proliferation, so that after 9 days in culture intricate networks of endothelial cells are formed and are stimulated by the addition of VEGFA and FGF2 (Robinson *et al.* 2008).

This study utilised this luteal cell culture system to investigate the roles of VEGFA, FGF2 and PDGF in luteal progesterone secretion and endothelial network formation by employing specific receptor tyrosine

kinase inhibitors to the activity of each of the growth factors. The hypotheses tested in the present study are a) FGF2 and VEGFA are critical for bovine luteal angiogenesis; and b) local luteal PDGF signalling stimulates the formation of endothelial networks.

Results

Formation of endothelial cell networks

Extensive formation of endothelial cell networks was observed in the control wells after 9 days in culture. The von Willebrand factor (VWF)-positive cells had developed into tubule-like structures and had formed highly organised, intricate networks that superficially resembled a capillary bed (Fig. 1A).

The degree of endothelial cell network formation was greatly reduced following treatment with all three angiogenic inhibitors; however, some VWF staining was present in all treatments (Fig. 1B–D).

Quantification of the VWF staining revealed that angiogenic inhibitors reduced the number of endothelial clusters ($P < 0.001$; Fig. 2A). The FGFR1 inhibitor SU5402 reduced the number of clusters by 94% versus controls ($P < 0.001$), with very few clusters present across the whole well. Both the VEGFR2 and PDGFR inhibitors, SU1498 and AG1295 respectively, resulted in lower numbers of endothelial networks compared to control wells ($P < 0.05$ and $P < 0.001$ respectively). Reductions were of a lesser extent than those following SU5402 treatment (Fig. 2A), with SU1498 and AG1295 treatment reducing cluster number by around 52 and 75% respectively.

The mean area of each endothelial cluster was affected by treatment overall ($P = 0.02$; Fig. 2B). While SU1498 and AG1295 had no effect on the mean size of individual endothelial clusters, inhibition of FGFR1 with SU5402 tended to reduce cluster size by ~40% ($P = 0.06$; Fig. 2B).

The total area of VWF staining was reduced by all angiogenic inhibitors ($P < 0.001$; Fig. 2C). SU5402 showed the greatest inhibition of endothelial network formation, with a >95% reduction in the total area of VWF staining compared with control wells ($P < 0.001$). Both SU1498 and AG1295 reduced total area of VWF staining by around 60 and 75% respectively ($P < 0.05$ and $P < 0.001$ respectively; Fig. 2C). While AG1295 consistently lowered VWF staining in each culture, the effects of SU1498 were more variable, with two cultures showing only a 25% reduction.

The total length of endothelial networks was reduced by angiogenic inhibitors ($P < 0.001$) in a similar manner to the total area of VWF staining (data not shown). SU5402 resulted in the greatest inhibition of endothelial network length, with a >95% reduction in total network length versus control wells ($P < 0.001$). SU1498 and AG1295 treatment reduced endothelial network length

Figure 1 The effect of angiogenic inhibitors on the formation of endothelial cell networks from dispersed luteal cells *in vitro*. Representative images of cells treated with (A) control medium containing 1 ng/ml VEGFA and 1 ng/ml FGF2, plus (B) 1 μ M SU5402 (FGFR1 inhibitor), (C) 2 μ M SU1498 (VEGFR2 inhibitor) or (D) 2 μ M AG1295 (PDGFR inhibitor). Endothelial cells were immunostained for von Willebrand factor (brown) after 9 days of culture. The arrows indicate the tubule-like structures and the scale bar represents 200 μ m.

by around 50 and 75% respectively ($P < 0.05$ and $P < 0.001$ respectively; data not shown). There was extensive cell proliferation in control and treated wells, with cells being confluent by 9 days.

Production of progesterone

Progesterone production increased eightfold from day 1 to 7, with intermediate values on day 5 ($P < 0.001$; Fig. 3) across all treatments. There were no further increases in progesterone production on day 9 in the control wells. Treatment with the VEGFR2 inhibitor SU1498 resulted in an overall decrease in progesterone production versus controls ($P < 0.001$). Specifically, progesterone production was similar to control wells on days 1 and 3, but was $\sim 25\%$ lower on days 5 and 7 ($P < 0.001$) following SU1498 treatment. However, by day 9, there were no differences when compared with control wells. By contrast, the production of progesterone in the presence of either AG1295 or SU5402 was not different from control wells at any time points ($P > 0.05$; Fig. 3). There was no interaction between treatment and time ($P > 0.15$).

Discussion

Using a new *in vitro* model of luteal angiogenesis, the present study showed that the inhibition of receptors for the proangiogenic mediators FGF2, VEGFA or PDGF all resulted in reduced endothelial network formation. Treatment with SU5402, an FGF2 signalling inhibitor, dramatically reduced endothelial network formation by around 95% versus controls, as a result of reductions in both the number of endothelial clusters and mean area of each cluster. Significant reductions in the total

area of VWF staining, and hence endothelial cell coverage, were also observed following treatment with SU1498 or AG1295 to block VEGFA and PDGF signalling respectively, although these reductions were less marked than following SU5402 treatment.

These results suggest that all three growth factors are active regulators of luteal angiogenesis. It also indicates that these factors must have complementary rather than redundant actions in luteal angiogenesis, since the absence of any one signal was sufficient to cause marked alterations in network formation, and the remaining factors were unable to compensate for the loss of any other factor. Studies of tumour angiogenesis have also shown that modulating FGF2 can inhibit the growth and vascularisation of xenografted tumours despite high levels of VEGFA (Czubayko *et al.* 1997).

Angiogenic regulators have been shown to act synergistically to stimulate angiogenesis. VEGFA and FGF2 in combination stimulate angiogenesis both *in vivo* and *in vitro* (Pepper *et al.* 1992, Asahara *et al.* 1995) and one underlying mechanism of co-stimulation is enhanced PDGFB–PDGFRB signalling (Kano *et al.* 2005). Indeed, the extent of the interplay between these factors may mean that target inhibition of one factor is almost equivalent to targeting the other(s).

Treatment with SU5402 resulted in the most dramatic reduction in endothelial clusters versus controls. Early studies suggested that FGF signalling is a key modulator of luteal angiogenesis, following the total inhibition of endothelial mitogenesis in response to ovine luteal-conditioned media that had been immunoneutralised for FGF2 (Grazul-Bilska *et al.* 1992). We have recently shown that luteal FGF2 concentrations are highest during early luteal development, further suggesting that FGF2 is a key mediator of the initiation of angiogenesis

Figure 2 The effects of angiogenic inhibitors on luteal endothelial network development *in vitro*. Luteal cells were dispersed from early bovine CL ($n=5$ cultures) and treated either with control medium containing 1 ng/ml VEGFA or 1 ng/ml FGF2 (Cont, open bars), plus 1 μ M SU5402 (FGFR1 inhibitor; dotted bars), 2 μ M SU1498 (VEGFR2 inhibitor; cross hatched lines) or 2 μ M AG1295 (PDGFR inhibitor; closed bars) for 9 days. Following image analysis and quantification of immunostaining for von Willebrand factor, the effects of inhibitors are shown on (A) mean number of endothelial clusters, (B) mean area of each endothelial cluster, and (C) total area of von Willebrand factor staining. For all three parameters, significant differences ($P<0.05$) between treatment groups are indicated by different letters $a<b<c$, and the values are mean $\pm$ S.E.M.

(Robinson *et al.* 2007) and in line with the observed reduction in network formation following SU5402 treatment. By contrast, others have shown that treatment to inhibit VEGFA bioactivity caused near absolute suppression of luteal angiogenesis in the rat, demonstrating that VEGFA is essential for neovascularisation (Ferrara *et al.* 1998). However, in our study inhibition of VEGFA only partially suppressed network formation and whether this may be due to species differences is currently unclear.

In the present study, the inhibition of PDGF signalling significantly reduced endothelial network formation in the absence of exogenous addition of ligand. AG1295 has been reported to completely prevent PDGF receptor autophosphorylation and DNA synthesis in response to PDGF stimulation, but exhibits

Figure 3 The effect of angiogenic inhibitors on the production of progesterone during culture in the *in vitro* luteal angiogenesis system. Dispersed luteal cells from early bovine CL ($n=6$ cultures) were cultured for 9 days, with media collected and replaced on days 1, 3, 5, 7 and 9. The cells were treated with control medium containing 1 ng/ml VEGFA and 1 ng/ml FGF2 (Cont, open bars), plus 1 μ M SU5402 (FGFR1 inhibitor; dotted bars), 2 μ M SU1498 (VEGFR2 inhibitor; cross-hatched lines) or 2 μ M AG1295 (PDGFR inhibitor; closed bars) for 9 days. There was a significant day ($P<0.001$; $a<b<c$) and treatment effect ($P<0.001$) with SU1498 decreasing progesterone production on days 5 and 7 ($***P<0.001$ versus CONT). However, neither SU5402 nor AG1295 treatment had any effect on progesterone production. The data are mean $\pm$ S.E.M.

only weak or negligible effects on the epidermal growth factor (EGF) receptor, insulin receptor, VEGF receptor or FGF receptor (Kovalenko *et al.* 1994). Given the specificity of PDGFR inhibition by AG1295 this suggests that PDGF is synthesised endogenously in our culture system. In support of this, PDGF ligands and PDGFRA were localised primarily to steroidogenic cells, while the PDGFRB was predominantly expressed by vascular cells in the rat CL (Sleer & Taylor 2007b), suggesting that the PDGF system is able to play a paracrine role in the CL.

Treatment with the tyrosine kinase inhibitors appeared to influence endothelial growth to differing degrees, with SU5402 showing the most marked reduction in total endothelial area. In addition, the nature of the inhibition seemed to differ, suggesting subtly different roles for each growth factor in modulating angiogenesis and luteal growth and development. FGF2, VEGFA and PDGF blockade all resulted in a reduction in the number of endothelial clusters formed in the luteal culture system. Following FGF2 inhibition there was a tendency for a reduction in the area of each cluster; a response that was not observed following VEGFA or PDGF inhibition. Furthermore, changes in endothelial networks in response to VEGFA inhibition were associated with significant reductions in progesterone production on days 5 and 7 of culture.

Alterations in the number of endothelial clusters might be the result of a reduction in an endothelial survival factor, as well as a block to proliferation. VEGFA not only stimulates proliferation of endothelial cells but it has also been shown to induce survival pathways (Gerber *et al.* 1998), including the expression of anti-apoptotic BCL2

(Nor *et al.* 1999). Similarly, FGF2 acts as a survival factor by suppressing endothelial cell apoptosis (Karsan *et al.* 1997).

A survival role for PDGF has also been suggested in bone endothelial cells (Langley *et al.* 2004). PDGF can also enhance endothelial cell survival indirectly, via the induction of VEGFA expression in pericytes which then acts on endothelial cells (Reinmuth *et al.* 2001). However, since the luteal cultures were supplemented with exogenous VEGFA, it is unlikely that PDGF signalling is acting via this indirect route. Pericytes lie within the basement membrane and envelop microvessels, in contact with the underlying endothelium and are important modulators of vascular structure and function (Armulik *et al.* 2005). Their roles include providing a physical scaffold, guiding the proliferating tips of newly sprouting vessels, and communicating with and between endothelial cells (Bergers & Song 2005). Previous experiments to block luteal PDGFRB signalling using an adenoviral approach induced luteal pericyte deficiency, with a reduction in pericyte coverage of >90% (Kuhnert *et al.* 2008). Interestingly, the inhibition of luteal pericyte recruitment was accompanied by a 45% reduction in microvessel endothelial cell density (Kuhnert *et al.* 2008). The results of the current study suggest that pericyte activation through PDGFRB signalling is important in stimulating luteal angiogenesis, thus highlighting the considerable intercellular communication and interdependency that exists between endothelial cells and pericytes (Armulik *et al.* 2005).

Treatment with SU5402 to inhibit FGF2 signalling tended to reduce endothelial cluster size versus controls, whilst SU1498 and AG1295 treatment appeared intermediate, and overall there was an effect of treatment on mean endothelial cluster size ($P=0.02$). The mean area of an endothelial cluster would likely be determined by coordinated actions on cell proliferation and migration. FGF2 and VEGFA are both known to be potent stimulators of endothelial cell mitogenesis and chemotaxis (Ferrara & Davis-Smyth 1997, Presta *et al.* 2005). PDGF can also stimulate chemotaxis (Kundra *et al.* 1994) and the migration of pericytes ahead of endothelial cells is thought to guide endothelial sprouts (Bergers & Song 2005).

The inhibition of VEGFA, FGF2 or PDGF action *in vivo* is associated with attenuated progesterone secretion resulting from changes in gross luteal structure, function and angiogenesis in the mouse, macaque and cow (Zimmermann *et al.* 2001, Fraser *et al.* 2005, Kuhnert *et al.* 2008, Yamashita *et al.* 2008). VEGFA and FGF2 stimulated progesterone release from microdialysed bovine early CL *in vitro* (Kobayashi *et al.* 2001). We have also shown previously that progesterone production was increased by the addition of FGF2 with VEGFA in the luteal angiogenesis culture system (Robinson *et al.* 2008). Similarly, the current study

demonstrates that the inhibition of VEGFA signalling by luteal cells *in vitro* can also reduce progesterone production, albeit to a small degree.

In conclusion, this study demonstrated that FGF2, as well as VEGF, both play important roles in regulating bovine luteal endothelial network formation *in vitro*. Moreover, we showed that even in the presence of VEGFA, FGF2 was critical for the formation of luteal endothelial networks and this emphasises the importance of FGF2 in controlling luteal angiogenesis. Furthermore, we established for the first time that local PDGF signalling stimulates the formation of endothelial networks *in vitro* in the cow. The precise regulation of luteal angiogenesis is likely to involve a complex interplay between these factors.

Materials and Methods

All materials were purchased from Fisher Scientific, Loughborough, UK unless stated.

Luteal angiogenesis culture system

The effects of angiogenic inhibitors on the development of bovine luteal endothelial cell networks were investigated using a physiologically relevant luteal angiogenesis culture system as described by Robinson *et al.* (2008). Briefly, coverslips (circular, 19 mm diameter $\times$ 0.25 mm thick, SLS, Nottingham, UK) were placed in a 12-well plate and coated with 1 ml of 10 μ g/ml fibronectin (Sigma) for 4 h, after which the fibronectin was removed and the wells were left to dry at 39 °C overnight. On the following day, the wells were rinsed with sterile distilled water, and then 1 ml endothelial cell media (ECM) was added to each well. The ECM consists of a specialised endothelial cell medium (EBM-2; Lonza, Wokingham, UK) to which gentamycin, heparin, human EGF, LR³-insulin-like growth factor 1, hydrocortisone and ascorbic acid were added as per the manufacturer's instruction. In addition, the medium was supplemented with 5 ng/ml LH (AFP11743B, biopotency 1.06 $\times$ oLH NIDDK-I-2; a gift from Dr A F Parlow, NIDDK, CA, USA), 100 units/ml penicillin, 10 μ g/ml streptomycin, 10 μ g/ml insulin, 5.5 μ g/ml transferrin, 5 ng/ml selenium (all Sigma) and 2% (v/v) fetal bovine serum (Lonza). Medium in all wells was further supplemented with 1 ng/ml VEGF and 1 ng/ml FGF2 (Lonza).

Tissue collection and luteal angiogenesis culture system

Bovine ovaries were collected from a local abattoir and transported to the laboratory in warmed 1 $\times$ PBS. In all experiments, only early CL (day 1–4) were used and these were selected based on previously outlined criteria (Ireland *et al.* 1980). The CLs were removed connective tissue trimmed away and then sliced up using scissors. Luteal tissue was dispersed by incubation in M199 medium containing 2 mg/ml collagenase I type 1A and 25 μ g/ml DNase I (all Sigma) for 2 $\times$ 45 min in a shaking water-bath at 37 °C. Cells (including luteal steroidogenic, endothelial, pericytes and fibroblasts) from

each incubation were pooled together and then filtered through a 70 µm cell strainer (BD Biosciences, Oxford, UK) and washed 3× in ECM. The cells were then plated out at 2×10^5 viable cells (as determined by trypan blue exclusion (Tennant 1964)) per well in a 12-well plate (total volume: 2 ml) and cultured at 39 °C in a humidified incubator in 5% CO₂/95% air. The medium was collected and replaced after 1, 3, 5 and 7 days of culture. On day 9, the medium was collected and the cells fixed in acetone:methanol (1:1) at 4 °C for 5 min. The endothelial cells were then immunostained using VWF as an endothelial cell marker in order to assess network formation over time.

Effect of angiogenic inhibitors on endothelial cell network formation and progesterone production

The luteal cells were prepared from early corpora lutea (five cultures; one CL per culture) and plated as described above. The cells were then treated with control medium (containing 1 ng/ml VEGF and 1 ng/ml FGF2), or plus 1 µM SU5402 (IC₅₀=10 µM, specific FGFR1 inhibitor), 2 µM SU1498 (IC₅₀=0.7 µM, specific VEGFR2 inhibitor) or 2 µM AG1295 tyrphostin (IC₅₀=0.5 µM, specific PDGFRA and PDGFRB inhibitor). SU5402 interacts with the catalytic domain of the FGFR1 (Mohammadi *et al.* 1997) and has been used in numerous studies to specifically block FGF signalling and FGF2-induced angiogenesis (Numata *et al.* 2006, Nicoli *et al.* 2009). SU1498 inhibits tyrosine phosphorylation of VEGFR2, resulting in the suppression of endothelial cell proliferation in response to VEGF, and an inhibition of angiogenesis (Strawn *et al.* 1996, Katanasaka *et al.* 2008). Tyrphostin AG1295 has been reported to abolish autophosphorylation of the PDGF receptors and to block PDGF-stimulated DNA synthesis in endothelial cells (Kovalenko *et al.* 1994). All inhibitors were purchased from Calbiochem, Nottingham, UK and were initially dissolved in DMSO. The final concentration of DMSO in each treatment was <0.1% (v/v). The control wells were treated with 0.1% (v/v) DMSO. Each treatment was performed in triplicate with the cells being treated continuously for 9 days. The concentrations of angiogenic inhibitors used were based on IC₅₀ values and previously reported concentrations (Banai *et al.* 1998, Kurimoto *et al.* 2004, Hatziapostolou *et al.* 2006, Sugiura *et al.* 2007, Wang *et al.* 2007). A further experiment was carried out to investigate the production of progesterone ($n=6$ additional cultures). This was performed as described above with media being collected on days 1, 3, 5, 7 and 9 for progesterone analysis.

Immunostaining of endothelial cells with VWF

After fixation and each step, the cells were washed twice with 1×PBS for 5 min each wash. The first step was to block endogenous peroxidase activity with 3% (v/v) hydrogen peroxide in methanol. Non-specific binding was blocked by incubating sections with 20% (v/v) normal goat serum (NGS; Sigma) for 30 min and with no washing step, the cells were incubated with 4 µg/ml polyclonal rabbit anti-human VWF (Dako, High Wycombe, UK) in 2% (v/v) NGS, as previously

validated (Robinson *et al.* 2008). The slides were incubated in a humidified chamber at 4 °C overnight. On the following day, the primary antibody was detected using the Vectorstain ABC method as per instructions (Vector Laboratories, Peterborough, UK) and visualisation was performed with diaminobenzidine (Vector Laboratories). The cells were counterstained with haematoxylin, dehydrated in graded IMS, then xylene. The coverslips were then removed from the well and mounted in distrene-plasticiser-xylene mountant.

Quantification of VWF immunostaining by image analysis

The quantification of VWF staining was performed as previously described (Robinson *et al.* 2008) with some minor modifications. All image analysis was performed using Image Pro-Plus 6.0 (Media Cybernetics, Wokingham, UK). The sections were visualised under a 5× objective lens and the area of brown (VWF) staining was highlighted. The highlighted area was then smoothed and any holes in the staining were removed. Only areas of VWF staining > 150 µm² and with tubule-like appearance were included in the analysis. The number and area of each cluster was recorded. The programme also determined the total area of VWF staining and total length of endothelial cell networks formed. This was repeated for a total of 20 fields of view across the whole well. Within each culture, 2 wells per treatment were randomly selected and analysed.

Progesterone analysis

The spent culture media was collected and stored at −20 °C for progesterone analysis. The progesterone concentration in media was determined using EIA (Ridgeway Science, St Briavels, Gloucestershire, UK) as previously validated (Picton *et al.* 1999). The samples were diluted 200–500-fold into 1×PBS as appropriate. All wells were analysed in duplicate. The intra-assay coefficient of variation (CV) was 8.3% and inter-assay CV at 4.3 ng/ml was 8.0%.

Statistical analyses

All data were checked for normality and heterogeneity of variance and were log transformed where appropriate. The number of endothelial clusters, mean area of each cluster and total area and length of VWF staining were analysed by randomised block one-way ANOVA. The variables were blocked by culture, with treatment as the factor. If a significant difference ($P<0.05$) was observed then Bonferroni's multiple comparison test was performed to elucidate where the differences lay. The production of progesterone was expressed as the concentration of progesterone in spent media per day. The production of progesterone was analysed by repeated measures ANOVA with day of culture as time factor and treatment as the factor. The data was blocked by culture. Bonferroni's multiple comparison test was performed on progesterone production on each day of culture to determine which treatments were different from the control.

Declaration of interest

A declaration of interest statement reporting no conflict of interest has been inserted. Please confirm whether the statement is accurate.

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