Equine Endometrial Explants Undergo Significant Degenerative Changes in Culture

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Running head: endometrial explant degeneration in culture

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ABSTRACT

The current study evaluated equine endometrial explants following 12, 24, and 48 hours in culture. Measurement of an indicator of cell death in explant supernatant, light microscopy, and gene expression of biomarkers of endometrial function and cellular stress, were used to compare the effect of six different media on explant viability and morphology. Viability of explants was assessed indirectly through measuring LDH activity in the culture supernatant. Regardless of culture medium composition, a significant increase in LDH activity was observed within 12 hours of culture, indicating occurrence of cell damage. Morphological analysis through light microscopy revealed degenerative changes occurring within 12 hours and after 48 hours there is nearly complete loss of luminal and superficial glandular epithelium and diffuse detachment of deep glandular epithelium. Transcript abundance of prostaglandin-endoperoxide synthase 2 (*PTGS2*), estrogen receptor 1 (*ESR1*), and vascular endothelial growth factor (*VEGF*) was assessed as biomarkers of endometrial function. A marked increase in *PTGS2* and *VEGF* expression occurred, *ESR1* displayed more or less steady expression levels. Above described changes were seen irrespective of cell culture medium used. The marked increase in expression in *PTGS2* expression presents a limitation to using endometrial explants in the current culture system to study aspects of endometrial function such as the inflammatory response to insemination.

Keywords: endometrium, mare, explant culture, degeneration

INTRODUCTION

Endometrial explants and primary cultures of endometrial epithelial and stromal cells have been used to study endometrial function *in vitro* in the mare. The endometrium is a complexly organized tissue and it is well known that endometrial function depends on the interplay between stromal and epithelial cells (Pierro et al., 2001). A limitation of primary cell culture is the loss of phenotype by cells; primary equine endometrial epithelial cells for instance lose the ability to secrete Prostaglandin F2α following their first passage (Szostek et al., 2012). Endometrial explants have been used as *in vitro* tool to study various aspects of equine endometrial function such as the effect of conceptus' secretions on prostaglandin release (Ealy et al., 2010) and the effect of inflammatory mediators (Penrod et al., 2013) and insemination (Nash et al., 2010) on prostaglandin release and gene expression. Endometrial explants are considered the closest approach to *in vivo* conditions since structural arrangement of luminal epithelium cells, glandular epithelial cells and stromal cells is preserved. Explants furthermore contain immune cells and endothelial cells, cell types contributing to endometrial function. Few studies, none of which were carried out in equine, have addressed limitations of endometrial explants in culture. Human endometrial explants have limited viability in vitro. Morphological integrity of stromal cells shows signs of disruption as early as 12 hours after culture (Bersinger et al., 2010) and after 24 hours of culture significant degeneration such as fragmentation of glands is visible upon light microscopy (Bersinger et al., 2010; Schafer et al., 2011). Within 6 hours of culture, transcript abundance of progesterone and estrogen receptor decreases, while expression of leukemia inhibitory factor and prostaglandin-endoperoxide synthase 2 increases (Schafer et al., 2011). Even though endometrial explants are commonly used to study endometrial function in the mare, the effect of in vitro culture on their functionality and integrity has not been assessed.

The objective of the current study was to critically assess equine endometrial explants in culture. Light microscopy, gene expression of biomarkers of endometrial function and cellular stress, and measurement of an indicator of cell death in explant supernatant were used to compare the effect of six different media on explant viability. It was hypothesized that degeneration of explants would occur irrespective of the composition of the culture medium used.

MATERIALS AND METHODS

Uterine explant culture

All animal procedures were completed in accordance with and with the approval of the institutional animal care committee at the University of Calgary. Uteri were collected from mares following euthanasia through captive bolt, transported to the lab on ice and opened longitudinally to remove endometrial tissue using tweezers and a scalpel (n=3). Endometrium was cut into smaller pieces (2 – 5 mm), two pieces were placed into a well of a 12-well culture plate (combined weight 35.9 ± 13.7 mg) and 1 ml of cell culture medium was added. Six different cell culture media were used; the base medium consisted of DMEM/F12 high glucose (Thermo Fisher Scientific, Burlington, ONT) supplemented with 1% antibiotic-antimycotic (Thermo Fisher Scientific; contains amphotericin B, penicillin, and streptomycin) and 2 mM glutamine (Thermo Fisher Scientific)and with the addition of serum as follows: (1) serum free (no serum added), (2) 1% FBS (1% fetal bovine serum (Thermo Fisher Scientific) added), (3) 10% FBS (10% fetal bovine serum added), (4) 1% CS (1% charcoal stripped fetal bovine serum added), (5) 10% CS (10% charcoal stripped fetal bovine serum added), and (6) 1% BSA (1% bovine serum albumin (Thermo Fisher Scientific) was added). Four replicates were set up and at 0, 12, 24, and

48 hours samples were collected; at each time point one piece of endometrium was frozen at minus 80ºC, the other piece of endometrium was fixed in 10% neutral buffered formalin (VWR, Mississauga, ONT), and the explant supernatant was frozen at minus 80ºC.

Lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was measured in explant supernatant using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Burlington, ONT). Explant supernatant was diluted 1:10 using sample buffer and the assay was carried out according to manufacturer's instructions. Absorbance was measured at 490nm and 680nm using EnVision Multilabel Reader (Perkin-Elmer, Waltham, Massachusetts); to determine LDH activity the 680nm absorbance value was subtracted from the 490nm absorbance value. The change in LDH activity of explant supernatant at 12, 24 and 48 hours was calculated relative to baseline (0 hours) to account for the fact that serum itself can contain LDH.

Histology

Based on the results of the LDH activity assay, explants cultured in 10% FBS (highest increase in LDH activity compared to baseline) and 1% CS (representative of 1% BSA, 1% CS, and serum free media which had less increase in LDH than 10% FBS) were processed for histological analysis. Following fixation in 10% neutral buffered formalin, endometrium samples were embedded in paraffin, and tissue sections (5 μm) were cut from the paraffin blocks. Hematoxylin and eosin stain (H&E stain) was carried out according to routine protocols and samples were assessed by a blinded, American College of Veterinary Pathologists-boarded veterinary pathologist. In brief, each section was examined for the integrity of endometrial

epithelial cells, detachment of endometrial epithelial cells from basement membranes and adjacent epithelial cells, stromal rarefaction, and nuclear fragmentation (indicating apoptosis or necrosis). Four components were assessed in each sample: endometrial luminal epithelial cells, superficial endometrial glands, deep endometrial glands and endometrial stroma.

Real-time RT-PCR

RNA from explants cultured in 10% FBS and 1% CS was isolated using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's recommendation and quantified via spectrophotometry using a NanoDrop ND-1000 (Agilent Technologies, Palo Alto, CA, USA). RNA samples (500 ng/reaction) were treated with RNase-free DNase I (Thermo Fisher Scientific) for 15 min at 37°C, heat denatured (75°C for 10 min) and then reverse transcribed using High Capacity cDNA Reverse Transcription Kit and random hexamer primers (Thermo Fisher Scientific). The mRNA expression of *GADD45B* (Gene ID: 100059768), *PMAIP1* (Gene ID: 100629496), *PTGS2* (Gene ID: 791253), *VEGF* (Gene ID: 100033839), *ESR1* (Gene ID: 791249), and *GAPDH* (Gene ID: 100033897) was measured by real-time RT-PCR. Primers specific for the selected transcripts were designed using NCBI (http://www.ncbi.nlm.nih.gov/pubmed). Real-time PCR was completed using FAST SYBR Green PCR Master Mix (Thermo Fisher Scientific) with the following cycling conditions: 95°C for 20 sec; 40 cycles of 95°C for 3 sec, 60°C for 1 min; 55–95°C for dissociation. Efficiency of amplification for each primer was assessed through the analysis of a series of 10-fold dilutions. Each PCR was performed in duplicate. Specificity of amplification was monitored by including non-reverse transcribed RNA reactions for each sample and by completing a dissociation analysis at the end of each real-time run to verify the amplification of a single product. Cycle

threshold (Cq) values were obtained through the auto Cq function. Changes in gene expression were calculated by mean threshold cycle (Cq) and then normalized for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) using delta (Δ)Cq. Primer sequences were as follows: *GADD45B* forward (5'-GGCTCCGTGGGAAGGTTT-3'), *GADD45B* reverse (5'-CCCAAGGGACAAAAATGCAA-3'), *PMAIP1* forward (5'- AAGAGCTCCTGCCGCTGTT-3'), *PMAIP1* reverse (5'-CGGCTGCCCAGACTTACG-3'), *GAPDH* forward (5'-TTGTCAAGCTCATTTCCTGGTATG-3'), *GAPDH* reverse (5'-GTTAGGGGGTCAAGTTGGGAC-3'), *VEGF* forward (5'-ACCTCACCAAAGCCAACACA-3'), *VEGF* reverse (5'-TTTCTCCGCTCTGAGCAAGG-3'), *ESR1* forward (5'-ACGATGCCACCAGACCATTT-3'), *ESR1* reverse (5'-CATGTGAACCAGCTCCCTGT-3'), *PTGS2* forward (5'-AAAAGATCCTGCCCCATCCG-3'), *PTGS2* reverse (5'-CTCGCTGCAAGTCGTTTGAC-3').

Data analysis

Absorbance data (LDH activity) were transformed in folds of change from baseline to 12, 24 and 48 hours after treatment to make them comparable regardless of baseline absorbance. Fold changes at different times were tested using an ANOVA initially for each of the media. Subsequently, an overall Factorial ANOVA was used to test for differences between different times (absorbance fold change at 12, 24 and 48 hours) and between media. Equality of variance was tested by Levene's test, whereas Sidak's test was used to perform Post HOC comparisons to test for differences among groups and time steps (Sokal and Rohlf, 1995). A similar approach was used to test differences in relative mRNA abundance, comparing the baseline expression before treatment (medium) to the expression at different times, without further comparing

different media. Statistical analyses were performed using IBM SPSS 22 (IBM Inc. Chicago, IL, USA).

RESULTS

Lactate dehydrogenase activity

Figure 1 depicts the change in LDH activity of explant supernatant from baseline (0 hours) to 12, 24 and 48 hours using different media. Overall, we detected significant differences between media on the variation of absorbance from baseline ($p<0.001$). 10% FBS had a significantly higher increase than 1% BSA, 1% CS, and serum free media ($p<0.05$). 10% CS displayed a significantly higher increase than 1% CS ($p<0.05$). No significant difference were detected between 1% BSA, 1% CS, 1% FBS, and serum free medium ($p>0.05$).

Explant supernatant contained significantly more LDH at 12 hours when compared to baseline irrespective of medium ($p<0.05$). Comparing 12, 24, and 48 hours, no significant difference was detected among the three time points for 1% BSA ($p>0.05$). For 1% CS and serum free medium, no significant differences were detected between the change in absorbance at 12 and 24 hours ($p>0.05$), while significant differences were detected between 12 and 48 hours ($p<0.05$). 1%FBS displayed an opposite pattern. The only one that showed a continuous trend in increased absorbance was the 10% FBS in which the changes from 12 hours were significant both after 24 and 48 hours ($p=0.004$ and $p<0.024$ respectively). Different superscripts indicate $p<0.05$ within treatment (Fig. 1).

Histology

Endometrial samples from both treatment groups showed similar degenerative changes over the 48 hour culture period (Fig. 2). Briefly, luminal epithelium and the epithelium lining deeper endometrial glands degenerated fairly rapidly, while the epithelium lining superficial endometrial glands was more robust, and stromal cells were the last to degenerate. Overall, these changes were slightly more severe in the samples cultured in 10% FBS than in 1% CS.

After 12 hours of culture the height of luminal epithelial cells was markedly and irregularly reduced, with extensive loss of apical microvilli and patchy areas where the epithelium had become simple squamous or had been lost entirely. Multifocally, luminal epithelial nuclei were fragmented (necrosis or apoptosis). Superficial endometrial glands were histologically normal but the epithelial lining of deeper glands had detached from the underlying basement membrane and, in the sample cultured in 10 % FBS only, from adjacent epithelial cells. Diffusely, the stroma surrounding endometrial glands was rarefied and expanded by clear space (fluid accumulation).

After 24 hours of culture luminal epithelial changes were similar to, but more extensive than those at 12 hours, with frequent nuclear fragmentation or condensation and sloughing of cells from the surface. This was more severe in the sample cultured in 1% CS. The epithelial cells lining superficial endometrial glands were reduced in height and showed loss of apical microvilli, moderate nuclear fragmentation and condensation, multifocal cytoplasmic hypereosinophilia (necrosis) and occasional sloughing. The majority of epithelial cells lining deep endometrial glands had detached from both the basement membrane and adjacent epithelial cells and were free in the glandular lumen. This was slightly more severe in the sample cultured in 10% FBS.

After 48 hours of culture the luminal epithelium was attenuated, ragged or absent. The epithelial cells lining superficial endometrial glands showed frequent detachment from both basement membranes and adjacent cells, with frequent nuclear fragmentation or condensation and cytoplasmic hypereosinophilia. The epithelial cells lining deep endometrial glands were nearly diffusely sloughed. Numerous stromal cells showed nuclear fragmentation or condensation.

Relative mRNA abundance

ESR1 expression did not show any significant different across different times for neither 1% CS ($p=0.09$) nor 10% FBS ($p=0.12$), whereas *VEGF* and *PTGS2* did for both media (Fig. 3). In particular, the expression of *VEGF* showed clear differences for explants in 1% CS between baseline (BL) and the three other time points (BL vs 12 hr, $p=0.002$; BL vs 24 hr, $p=0.029$; BL vs 48 hr, $p=0.044$), but not between 12, 24 or 48 hours (all $p>0.05$). The expression of *VEGF* in 10% FBS was significantly higher at 12 hr ($p<0.001$) and 24 hr ($p<0.044$) when compared to BL. The expression of *PTGS2* was significantly increased at all time points when compared to baseline for both 1% CS (BL vs 12 hr, $p=0.007$, BL vs 24 hr, $p=0.001$; BL vs 48 hr, $p=0.005$) and 10% FBS ($p<0.001$). *PMAIP1* mRNA abundance was significantly increased at all time points when compared to baseline for 1% CS (<0.01), but not for 10% FBS. *GADD45B* and *RFXP1* were not expressed consistently by all samples and therefore no differential expression analysis was carried out for those transcripts (Table 1).

DISCUSSION

The current study evaluated equine endometrial explants following 12, 24, and 48 hours in culture. Measurement of an indicator of cell death in explant supernatant, light microscopy, and gene expression of biomarkers of endometrial function and cellular stress, were used to compare the effect of six different media on explant viability and morphology. Size of the explants (two pieces, combined weight 35.9 ± 13.7 mg) was chosen to resemble explant size commonly cited in the literature which ranges from 20 mg to 150 mg.

Viability of explants was assessed indirectly through measuring LDH activity in the culture supernatant. LDH is a cytoplasmic enzyme, which is released from cells following plasma membrane damage and its activity in culture supernatant therefore correlates to degree of cell damage in an explant culture system. Regardless of culture medium composition, a significant increase in LDH activity was observed within 12 hours of culture, indicating occurrence of cell damage. Explants cultured with no serum, 1% BSA, 1% CS, and 1% FBS displayed similar LDH activity across time points analyze; albeit not significant, 1% CS presented the least amount of increase in LDH activity. Explants culture in 10% FBS released significantly more LDH than those cultured with no serum, 1% BSA, and 1% CS. For further analysis, only explants culture in 1% CS (lowest degree of cell death) and 10% FBS (highest degree of cell death) were used.

Morphological analysis through light microscopy revealed similar degenerative changes over the 48 hour culture period. Luminal epithelium and the epithelium lining deeper endometrial glands degenerated fairly rapidly, while the epithelium lining superficial endometrial glands was more robust, and stromal cells were the last to degenerate. At 12 and 24 hours degenerative changes were slightly more pronounced in samples cultured in 10% FBS,

which is in agreement with the results of the LDH activity in the culture supernatant. Similar degenerative changes have been described for human endometrial explants in culture; after 24 hours of culture, significant loss of tissue integrity was observed in both serum-free and serum supplemented culture medium (Schafer et al., 2011). Likewise, based on scanning electron microscopy, disruption of morphological integrity was documented within 12 hours of in vitro culture (Bersinger et al., 2010).

Gene expression of growth arrest and DNA-damage-inducible, beta I (*GADD45B*) and phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*) was assessed as markers of cellular death. At 12 hours, *GADD45B* was expressed by all samples, thereafter however, *GADD45B* was not consistently expressed by all samples, with no clear pattern of appearance and disappearance of the transcript. *GADD45B* belongs to a family of stress-induced genes and is implicated in growth arrest and apoptosis in response to environmental stresses (Takekawa and Saito, 1998). Expression of *PMAIP1* increased only when explant were cultured in 1% CS. *PMAIP1* is a mediator of p53-induced apoptosis (Oda et al., 2000). Since expression was not significantly altered in explants cultured in 10% FBS, it is possible that a different mediator of apoptosis mediates the degenerative changes observed. Alternatively, explants cultured in 10% FBS may have undergone necrosis rather than apoptosis. Cell death by means of necrosis does not involve gene expression and is a passive, externally driven process (Hung and Chow, 1997).

Transcript abundance of prostaglandin-endoperoxide synthase 2 (*PTGS2*), estrogen receptor 1 (*ESR1*), and vascular endothelial growth factor (*VEGF*) was assessed as biomarkers of endometrial function. Release of PGF2α is a hallmark of the endometrial cells; *PTGS2* is a key enzyme in prostaglandin biosynthesis and converts arachidonic acid to prostaglandin endoperoxide H2 (PGH2). Regardless of culture medium, a marked, on average 22.6-fold

increase in *PTGS2* expression was observed. The marked increase in expression in *PTGS2* expression presents a limitation to using endometrial explants in the current culture system to study aspects of endometrial function such as the inflammatory response to insemination. Whereas the endometrium *in vivo* responds to insemination with increased release of PGF2α, no such increase could be observed when exposing endometrial explants obtained from mares in heat to semen (Nash et al., 2010). The already high expression of *PTGS2* might explain why explants fail to mimic the *in vivo* response to semen. Endometrial explants have been used successfully to study maternal recognition of pregnancy in the mare. It was demonstrated that conceptus-derived factors present within conceptus-conditioned medium reduce the expression of *PTGS2*, whereas oxytocin increases *PTGS2* transcript abundance (Ealy et al., 2010). Given the marked up-regulation of *PTGS2* following a short time in culture, endometrial explant, while suited, might not be an ideal system to study regulation of prostaglandin synthesis in the context of maternal recognition of pregnancy. Endometrial function is largely regulated via the actions of steroid hormones, namely estrogen and progesterone. *ESR1* expression was moderately reduced following culture in 1% CS for all time points, whereas 10% FBS displayed altered expression levels, which however did not show any significance. Steady expression levels of *ESR1* are important when studying estrogen-responsive genes and indeed explants can be used to study the regulation of estrogen-responsive genes such as Milk fat globule-EGF factor 8 (unpublished observation). *VEGF* plays a role in plays an important role in endometrial angiogenesis (Silva et al., 2011). Increased expression as a sequel to hypoxia has been reported for human endometrial cells in culture; given the three-dimensional structure of explants an increase in expression following *in vitro* culture can be expected.

To conclude, endometrial explants show a considerable degree of degeneration following *in vitro* culture. Analysis of gene expression markers of endometrial function show that *PTGS2* expression is markedly upregulated which poses limitations for using endometrial explants when studying inflammatory processes or certain aspects of early pregnancy.

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TABLE & FIGURE LEGENDS

Table 1 Transcripts *GADD45B* and *RFXP1* were not expressed consistently by all samples.

The number of samples out of n=3 displaying detectable mRNA levels following real-time RT-PCR are shown.

Figure 1: **Change in LDH activity of explant supernatant from baseline to 12, 24 and 48 hours using different media.** DMEM/F12 was supplemented with (1) serum free (Serum Free), (2) 1% BSA, (3) 1% charcoal stripped FBS, 1% CS, (4) 1% FBS, (5) 10% CS, and (6) 10% FBS. Boxplots with median (thick line), interquartile distance (box) and extreme values (whiskers) are reported. Different superscripts indicate $p<0.05$ within treatment.

Figure 2: Histologic detail of endometrial explants showing degenerative changes that take place over a 48 hour culture period. After 12 hours luminal epithelial cells are reduced in height and multifocally lost. Endometrial stroma is rarefied (edema). Endometrial glands show degenerative changes, including nuclear fragmentation and cellular detachment. After 24 hours both luminal and superficial glandular epithelia are markedly degenerate and there is extensive detachment of deep endometrial glands. After 48 hours there is nearly complete loss of luminal and superficial glandular epithelium and diffuse detachment of deep glandular epithelium.

Figure 3: **Changes in relative transcript abundance (delta Cq, dCq) of *PMAIP1, PTGS2*, *ESR1*, and *VEGF*) in explants cultured in different media.** DMEM/F12 was supplemented with 1% CS and 10% FBS. Scatter dot plot (mean with standard deviation) are depicted. dCq is inversely related to relative transcript abundance. Different superscripts indicate $p<0.05$ within treatment.

transcript	medium	0 hr	12 hr	24 hr	48 hr
RFXP1	1% CS	3/3	3/3	2/3	1/3
	10% FBS	3/3	3/3	3/3	0/3
GADD45B	1% CS	1/3	3/3	2/3	0/3
	10% FBS	1/3	3/3	1/3	2/3

Table 1 Transcripts *GADD45B* and *RFXP1* were not expressed consistently by all samples. The number of samples out of n=3 displaying detectable mRNA levels following real-time RT-PCR are shown.

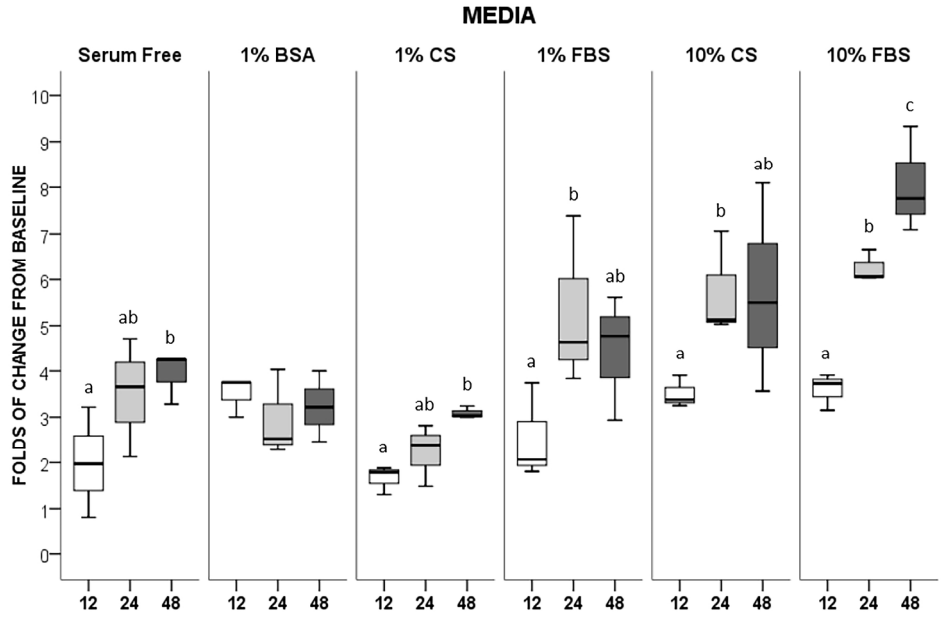

Figure 1 - Change in LDH activity of explant supernatant from baseline to 12, 24 and 48 hours using different media. DMEM/F12 was supplemented with (1) serum free (Serum Free), (2) 1% BSA, (3) 1% charcoal stripped FBS, 1% CS, (4) 1% FBS, (5) 10% CS, and (6) 10% FBS. Boxplots with median (thick line), interquartile distance (box) and extreme values (whiskers) are reported. Different superscripts indicate $p<0.05$ within treatment.

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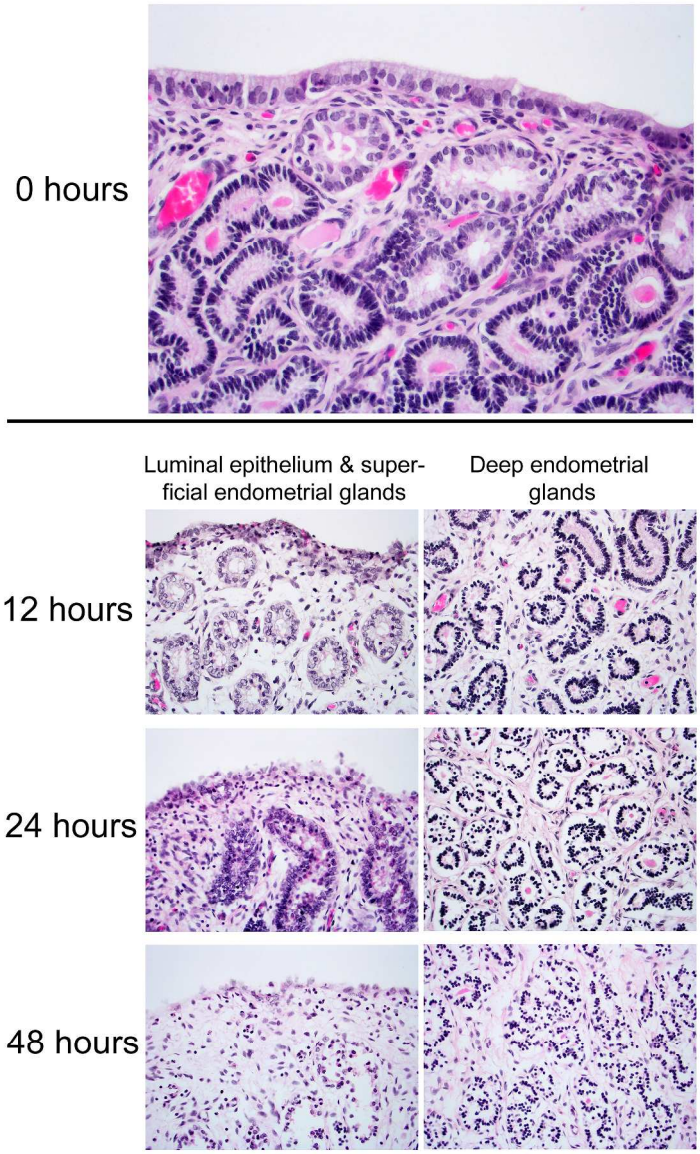

Figure 2 - Histologic detail of endometrial explants showing degenerative changes that take place over a 48 hour culture period. After 12 hours luminal epithelial cells are reduced in height and multifocally lost. Endometrial stroma is rarefied (edema). Endometrial glands show degenerative changes, including nuclear fragmentation and cellular detachment. After 24 hours both luminal and superficial glandular epithelia are markedly degenerate and there is extensive detachment of deep endometrial glands. After 48 hours there is nearly complete loss of luminal and superficial glandular epithelium and diffuse detachment of deep glandular epithelium.

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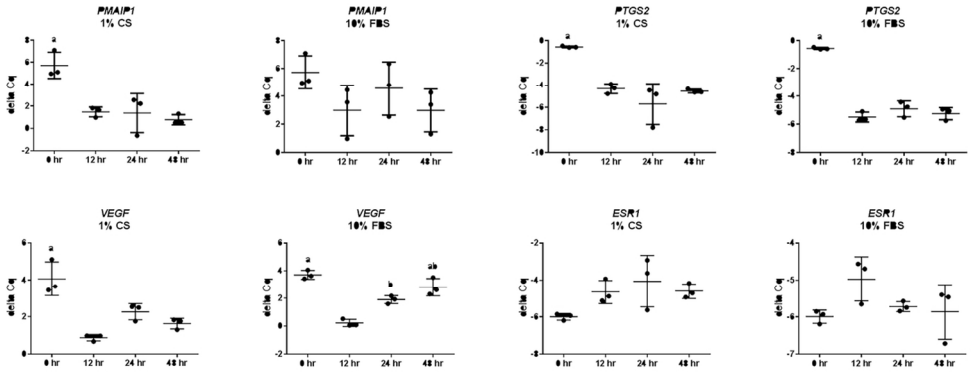

Figure 3 - Changes in relative transcript abundance (delta Cq, dCq) of PMAIP1, PTGS2, ESR1, and VEGF) in explants cultured in different media. DMEM/F12 was supplemented with 1% CS and 10% FBS. Scatter dot plot (mean with standard deviation) are depicted. dCq is inversely related to relative transcript abundance. Different superscripts indicate $p<0.05$ within treatment.

57x22mm (600 x 600 DPI)