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Intrauterine *Candida albicans* infection causes systemic fetal candidiasis with progressive cardiac dysfunction in a sheep model of early pregnancy

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ABSTRACT

INTRODUCTION: Several recent studies have identified a potential role for intrauterine *Candida albicans* in adverse pregnancy outcomes, including preterm birth. There is, however, a limited understanding of the impact of intrauterine candida infection on fetal well-being in early pregnancy. Using a sheep model of early pregnancy, the aims of this study were to determine: **i)** the ability of experimentally-induced intrauterine *C. albicans* to infect the fetus; **ii)** if *C. albicans* exposure in early pregnancy is associated with alterations in fetal cardiac function, as measured by spectral Tissue Doppler Imaging analysis of fetal cardiac function.

METHODS: Merino ewes carrying singleton pregnancies at 89 days gestation (term is ~150 days) received *C. albicans* (n=8) via ultrasound-guided intraamniotic injection. Saline-exposed fetuses served as controls (n=6). Spectral Tissue Doppler Imaging echocardiography and amniotic fluid collection was performed at baseline, 24 and 72 hours intrauterine *C. albicans* injection. Fetal tissues were collected at post-mortem for analysis of infection and inflammation.

RESULTS: Relative to saline control, intrauterine *C. albicans* infection resulted in pronounced increases in amniotic fluid TNF-α; ($p<0.05$) and cytokine / chemokine mRNA (IL-1β, IL-6, TNF-α and MCP-1; $p<0.05$) in the fetal myocardium, lung, skin and liver at 72 and 96 h post-infection. Spectral tissue Doppler imaging showed diastolic dysfunction at 24 h and severe biventricular diastolic dysfunction 72 h post-infection.

CONCLUSIONS: Intrauterine *C. albicans* infection in a sheep model of early pregnancy causes systemic fetal candidiasis, which is associated with a robust systemic inflammatory response and progressive cardiac dysfunction detectable by spectral Tissue Doppler Imaging.

INTRODUCTION

With advances in perinatal care, infants born early preterm (<32 weeks' gestation) frequently survive to reach adulthood, and up to 1 in 10 young adults will now have been born preterm (<37 weeks' gestation). Preterm birth has a number of different aetiologies, but intrauterine inflammation is a common clinical finding. Infection is frequent, occurring in up to 60% of early preterm births (<32 weeks gestation)[1]. Other causes of preterm birth (e.g. intrauterine distension and hemorrhage) are also mediated, at least in part, through an inflammatory response. A significant body of literature serves to demonstrate a strong association between intrauterine infection caused by a number of microorganisms including *Ureaplasma*, *Fusobacterium*, *Prevotella, Bacteroides,* and *Streptococcus* spp. and preterm birth.[2,3] There is also an emerging body of evidence to suggest that intrauterine infection caused by *Candida albicans*, is associated with preterm birth and adverse perinatal outcomes. *C. albicans* has been detected in amniotic fluid samples from cases of spontaneous preterm birth, and intrauterine *C. albicans* is associated with fetal death and sub-optimal neuro-development.[4,5] Interestingly, the fetal responses to *C.albicans* in fetal sheep in mid-late pregnancy[6] (minimal chorioamnionitis in association with profound fetal inflammatory responses and candidiasis) are distinct from those driven by *Escherichia coli* lipopolysaccharides (LPS)[7,8] or *Ureaplasma parvum* serovar 3.[9] These data suggest that the intrauterine response to infection, and thus the potential short- and long-term effects on infant well-being may have some degree of organism specificity.

Being born early conveys a potential for detrimental effects on a number of organ systems, including the central nervous system, lungs and cardiovascular system.[10] Children and adults born preterm have an increased risk of death compared with those who were born at full term, with a 2-fold risk of mortality in young adults born extremely preterm (<28 weeks gestation).[11] Deaths from cardiovascular disease contribute to this mortality, with the risk inversely proportional to gestational age at birth. Furthermore, prematurity has been linked to cardiovascular risk factors such as hypertension, abnormal glucose regulation and

cerebrovascular disease.[12,13,14] The mechanisms underlying this increased cardiovascular risk are not clear, but recently published studies using cardiac magnetic resonance imaging have revealed profound differences in cardiac function associated with preterm birth.[15,16] These findings in adults are consistent with observations that left ventricular mass increases more in the first month of life in preterm infants than those born at term.[17] In sheep, abnormal maturation and accelerated postnatal cardiomyocyte hypertrophy has been related to preterm birth.[18]

A recent study found that cardiomyocyte growth in the fetal heart was markedly affected by *in utero* inflammation in a sheep model of pregnancy.[19] This study also showed that intrauterine inflammation resulted in compromised cardiac performance, vulnerability to damage and arrhythmias in *ex vivo* experiments. Other studies have shown cardiac dysfunction in association with chorioamnioitis in human pregnancy,[20] and in response to intra-amniotic LPS in mice.[21] Together, inflammation and preterm birth can result in cardiac dysfunction and vulnerability to ischemic injury that may persist through adult life.

With continued high preterm birth rates in developed countries (7-13%), long-term cardiovascular health effects will have an increasing clinical and public health impact. Understanding the effects of intrauterine infection on the fetus is crucial to developing strategies to mitigate the impact on fetal well-being, as well as to allow for the development of novel diagnostic and preventative approaches. We have previously demonstrated that intrauterine exposure to *E.coli* LPS, *U. parvum* and *C. albicans*, generates distinct inflammatory responses in fetal sheep at early and late gestations.[6, 17] To date, however, our ability to perform direct evaluation of the effects of intrauterine infection and inflammation on fetal cardiac function, and thus correlate real-time functional changes in fetal physiology with changes in infection status and intrauterine inflammation, have been restricted by limitations in the ability of imaging technologies to resolve functional changes in the small, rapidly pulsing fetal heart. Furthermore, although the fetal immunological capacity changes

markedly across gestation[22], there have been very few studies in clinically relevant, early gestation models of intrauterine infection and inflammation.[23] With recent methodological advances spectral Tissue Doppler Imaging (TDI) of the fetal heart is now both feasible and reliable in human pregnancy.[24] In the present report, we used a well-validated sheep-model of early pregnancy and aimed to determine: **i)** the ability of experimentally-induced intrauterine *C. albicans* to infect the fetus; **ii)** the fetal capacity to mount an inflammatory response to *C. albicans* exposure; and **iii)** if *C. albicans* exposure in early pregnancy is associated with alterations in fetal cardiac function, as measured by spectral tissue Doppler imaging of fetal cardiac function.

METHODS AND MATERIALS

Animal model

Animal studies were approved by The University of Western Australia's Animal Ethics Committee (RA/3/100/1289). All animal work was performed in Perth, Western Australia and complied with the National Health and Medical Research Council Australian Code for the care and use of animals for scientific purposes.[25] Sheep were housed in a custom-built sheep research facility that allows sheep to remain in established flock structures, in well-contained paddocks with access to shelter.

All intrauterine interventions were performed under ultrasound guidance as previously published[8]. Briefly, the wool covering the abdomen was clipped close to the skin and then thoroughly cleaned with an alcohol / chlorhexidine solution (non-irritant) and then coated in iodine. The fetus and amniotic cavity were visualised by ultrasound and amniocentesis performed under direct ultrasound guidance. Procedures took less than two minutes on average.

For the *C. albicans* group, date-mated merino ewes each carrying a single fetus were sampled for amniotic fluid and received a single intra-amniotic injection of 10^7 colony-forming units (CFU) of *C. albicans* in 2 mL sterile saline under ultrasound guidance (n=8). For the saline control group, date-mated merino ewes each carrying a single fetus were sampled for amniotic fluid and received a single intra-amniotic injection of 2 mL sterile saline under ultrasound guidance (n=6).

Amniotic fluid placement was confirmed by the presence of chloride in samples prior to injection of either *C. albicans* or sterile saline, using a Rapidlab 1265 blood gas analyser (Siemens, Australia). For *C. albicans*-exposed animals, ultrasound assessment of fetal cardiac function (see below) and amniotic fluid collection was performed prior to, 24 and 72 h after intraamniotic injection of 10^7 CFU *C. albicans*. Animals in the *C. albicans* group were

euthanized by intravenous injection of pentobarbitone (160 mg/kg) and the fetus surgically delivered either 72 (n=4) or 96 (n=4) h after intraamniotic infection was established. Animals in the saline control group were euthanized by intravenous injection of pentobarbitone (160 mg/kg) and the fetus surgically delivered. Gestational age at delivery was 94-95 d for both *C. albicans* and saline control groups.

Amniotic fluid, cerebrospinal fluid and tissues for mRNA expression analyses were collected at necroscopy and snap frozen in liquid nitrogen. Tissue (fetal myocardium, skin, chorioamnion, liver and lung) from 72 and 96 h *C. albicans* animals were pooled for cytokine mRNA and histological analyses. Previous experiments in mid-late pregnancy demonstrated *C. albicans* infection caused significant fetal compromise; accordingly, the length of infection was staggered as the investigators were uncertain *a priori* as to the length of time a comparatively immature fetus might survive exposure to intrauterine *C. albicans*.

Fetal blood (2 mL) was collected from the umbilical cord and inoculated into a BACTEC™ Peds Plus culture vial (Becton Dickinson, Franklin Lakes, NJ) for microbiological culture. Fetal lung (right upper lobe) for histological analysis was perfusion fixed (30 cmH_20) in 10% neutral buffered formalin for 24 h before paraffin embedding. Fetal skin tissues for histological analysis were cryo-preserved in optimum cutting temperature (OCT) compound (Sakura Finetek, the Netherlands). 5-6 animals were analysed from each group.

Candida albicans culture for intra-amniotic injection

A single clinical isolate of *C. albicans* was cultured on Difco™ Sabouraud Dextrose Agar (Becton Dickinson) at 37°C for 48 h. Single colonies were inoculated into sterile 1 x phosphate-buffered saline (PBS) (Sigma-Aldrich, St Loius, MO). *C. albicans* colony morphology was confirmed by growth on Brilliance Candida Agar (Oxoid, Adelaide, Australia). Inoculums were quantified using a plate dilution series as per standard microbiological methods and recorded as CFU/mL. Quantified inoculums (10^7 CFU in 2 mL

1 x PBS) were stored at −80 °C prior to use.

Nucleic Acid Extraction

Total RNA was extracted from liquid nitrogen–homogenized fetal tissues using TRIzol as previously reported.[26] Extracted RNA was treated with Turbo-DNase (Life Technologies, Carlsbad, CA) to remove any residual DNA and subsequently quantified on a Qubit 2.0 fluorometer (Life Technologies) using a broad-range RNA quantitation kit (Life Technologies). RNA yields from fetal tissues were normalized to 100 ng/µL using nuclease-free water (Life Technologies).

Candida albicans detection

The presence of viable *C. albicans* in amniotic fluid samples was determined using a Sabaraud-Dextrose agar plate dilution series as described above. Three single colonies from positive plates were subsequently inoculated onto Candida Brilliance agar (Oxoid) for confirmation of isolate identification. The presence of viable *C. albicans* in fetal blood samples collected at post-mortem was determined by incubation of 2 mL of fetal blood in BACTEC™ Peds Plus culture vials at 37°C for up to 96 h. At 24 h intervals, a 100 µL sample of blood culture was aseptically drawn from the culture vials and inoculated onto 5% sheep blood agar and Sabaraud-Dextrose agar. Positive cultures were confirmed as *C. albicans* by inoculation of three single colonies onto Candida Brilliance agar (Oxoid).

Histology

Fetal skin was dissected, embedded in OCT and immediately frozen on dry ice before thin (9 µm) cryo-sectioning. Immunofluorescence staining for the identification of hyphal structures in the epidermis was performed with rabbit anti-*C. albicans* (Meridian Life Sciences, Memphis, TN) and Alexa Fluor 488 anti-rabbit IgG antibodies (Life Technologies) diluted in 1x PBS containing 0.1% Tween 20 (Life Technologies) and 5% fetal calf serum to 1:600 and

1:10,000, respectively. Sections were blocked for 2 h in 1 x PBS containing 5% fetal calf serum at 4°C in a humidified chamber. Sections were incubated with primary antibody overnight at 4°C in a humidified chamber and secondary antibody for 2 h at room temperature in a darkened humidified chamber with washing between steps as appropriate. Washed sections were coated in Vectashield with DAPI (Vector Laboratories, Burlingame, CA), cover-slipped and sealed. Imaging was performed on a Zeiss LSM510 microscope (Carl Zeiss Microscopy, Oberkochen, Germany) using an EC Plan-Neofluor 20 x objective (Carl Zeiss Microscopy). Skin and chorioamnion sections were stained with haematoxylin and eosin as previously described.[27]

Enzyme-Linked Immunosorbent Assays (ELISA)

Quantification of TNF-α protein concentrations in amniotic fluid and arterial cord blood plasma was performed using Vetset ELISA kits (VS0285B-00. Kingfisher Biotech Inc., St. Paul, MN). Standards (run in triplicate) and samples (run in duplicate) were incubated for 16 h at 4°C before the assays were performed in accordance with manufacturer's instructions. 100 µL TMB substrate (T8865; Sigma Aldrich, St. Loius, MO) was added to each well and the plate incubated in the dark at room temperature for 15 min. The chromogenic reaction was halted by the addition of 100 µL 2% sulfuric acid solution in 1 x PBS (Sigma) to each well. The plate was then immediately read at $\lambda = 450$ nm.

Relative quantification of mRNA expression

Ovine-specific PCR primers and hydrolysis probes for interleukin (IL)-1β, IL-6, IL-8, IL-17, TNF-α, MCP-1, SMAP-29, SBD-1, and SBD-2 (Life Technologies) were used to perform quantitative PCR reactions on RNA from fetal myocardium, skin, chorioamnion, liver and lung tissue. Reactions were performed using an EXPRESS One-Step SuperScript qRT-PCR kit (Life Technologies) with 25 ng template RNA in a total volume of 20 µL as follows: 1 x 15 min reverse transcription at 50 °C and an initial denaturation at 95 °C for 20 s, followed by

40 cycles of 95 °C for 3 s and 60 °C for 30 s. All reactions were performed in 96-well plates on a ViiA7 real-time PCR thermocycler (Life Technologies). Cq values were normalized to 18S rRNA and expressed as fold changes relative to pooled control values. Statistical analyses were performed on dCq values.

Ultrasound imaging and analysis

Ultrasound assessment was performed by one operator with ewes held by an experienced handler in a dorsal recumbent position similar to that used during shearing. Measurements were taken using a Philips CX50 system with an S5-1 phased array probe (both Philips Healthcare, the Netherlands) with TDI program set to pulsed-wave Doppler mode. The ultrasound beam was focused parallel to the longitudinal myocardial wall and ventricular myocardial velocities were measured from apical or basal four-chamber views. Doppler sample volume was placed at the level of the lateral mitral annulus, at the basal level of the inter-ventricular septum, and the level of the lateral tricuspid annulus. Data were stored in cineloop format, and analysed at St George's University, London, by an investigator blinded to treatment.

The TDI data were used to derive cardiac indices of myocardial motion in systole and diastole using offline analyses (QLAB 10; Philips Healthcare). Annular peak velocity profiles and their peak values were obtained in systole (S'), early diastole (E'), and atrial contraction (A'). E'/A' indices were calculated. Criteria for image acquisition were applied as follows: maximum sweep speed (15 cm/s); high-pass wall motion filter (WMF) setting; reduced gain in order to observe echogenic valve clicks and hypoechogenic LV inflow and outflow waveforms. E/A spectral waveforms were visualized as positive in the apical chamber view, or as negative in the basal chamber view.

Statistics

Spectral TDI data descriptive statistics are presented as mean and standard deviation. The

Wilcoxon test was performed to test normality. Normally distributed data were analysed with repeated measure ANOVA to test the null hypothesis that there was no difference in the means between 0, 24 and 72 h values. Cytokine mRNA and protein concentration data were assessed for normality with Shapiro-Wilk tests. Parametric data were tested for group mean differences using one-way ANOVA. Data were analysed using IBM SPSS for Windows, Version 20.0 (IBM Corporation, Armonk, NY).

RESULTS

Necroscopy data

One animal from the *C. albicans* group (96 h infection) was found dead at delivery and excluded from analyses. All other animals were delivered alive. There was no significant difference in delivery weight, arterial cord blood pH, pO_2, or pCO_2 (data not shown). Intrauterine *C. albicans* infection was confirmed by culture, and hyphae were directly visualized in fetal skin in haemotoxylin and eosin stained sections (Figure 1, panel ii, B). No hyphal structures were identified in skin from control animals (Figure 1, panel ii, A). Identification of hyphae as *C. albicans* was by immunohistochemistry in treated animals (Figure 1, panel i, A-C). At least 6/7 (one sample was not collected) animals from the *C. albicans* group had positive blood cultures for *C. albicans*. 6/7 animals from the *C. albicans* group had positive cerebrospinal fluid *C. albicans* cultures at delivery. The average *C. albicans* amniotic fluid culture count in animals delivered at 96 h was 2.2×10^6 CFU/mL. No saline (n=4; two samples were not collected) or *C. albicans* (n=8) animals had positive baseline amniotic fluid *C. albicans* cultures.

Cytokine expression

Concentrations of TNF-α (Figure 2) were increased in the amniotic fluid in *C. albicans*-exposed animals at 72 h compared to control samples collected at delivery. Relative to saline control, increased TNF-α protein concentration was also identified in cord blood plasma samples from *C. albicans*-infected animals collected at delivery (0.1±0.09 vs. 0.02±0.03

ng/mL, $p<0.05$). Fetal lung (right upper lobe) from *C. albicans*-infected animals showed profound airspace consolidation (Figure 1, panel iii, A), whereas saline treated controls had no evidence of consolidation (Figure 1, panel iii, B).

Large relative increases in cytokine / chemokine transcript expression were detected in the fetal lung, and liver (Figure 3 A and B, respectively; $p<0.05$). mRNA levels for inflammatory genes *IL-1β, IL-6, TNF-α* and *MCP-1* were increased in the myocardium of *C. albicans* treated animals when compared controls receiving normal saline (Figure 3, C). Limited changes in cytokine mRNA expression were detected in the fetal skin, with only transcripts for *IL-1β* (mean 2.8-fold increase) and *MCP-1* (mean 4.3-fold increase) significantly ($p<0.05$) elevated relative to saline control. There was no statistically significant change in the mRNA transcripts for *IL-1β, IL-6, IL-8, TNF-α* or *MCP-1* in chorioamnion tissues (data not shown).

Tissue Doppler Imaging

Results of cardiac TDI examination of the inter-ventricular annulus at 0 (pre-infection), 24 and 72 h following inoculation of *C. albicans* are summarized in Table 1 and Figure 4. After 24 h, there was evidence of diastolic dysfunction with a significant decrease in interventricular septum peak annular early diastolic velocity (IV E'; 26% decrease; $p=0.018$) and early diastolic/late diastolic ratio (IV E'/A'; 31% decrease; $p=0.016$). At 72 h there was evidence of increased biventricular systolic function, with a significant increase in mitral valve peak systolic velocity (MV S'; 32% increase; $p=0.022$) and interventricular septum peak systolic velocity (IV S'; 25% increase; $p=0.050$). These changes were associated with the finding of diastolic dysfunction in both MV (abnormal relaxation) and TV (pseudo-normal) indices (IV A'; 34% increase; $p=0.023$ and TV E' 21% increase; $p=0.019$).

COMMENT

The primary findings of this study are: **i)** that intrauterine *C. albicans* infection in early pregnancy rapidly involves the fetus, causing systemic infection (blood, tissues and cerebrospinal fluid) characterized by hyphal invasion of the epidermis and dermis, marked alveolar consolidation and profound inflammatory changes in fetal, but not chorioamnion tissues; **ii)** that the magnitude of changes in fetal inflammatory mediator expression was greatest in the lung and liver, but comparatively mild in the heart and **iii)** that intrauterine *C. albicans* infection is associated with fetal cardiac dysfunction that is detectable by spectral TDI.

These results should be considered with reference to the experimental design. A strength of our study is the use of a large animal model of early gestation pregnancy, which is relevant to clinical practice as infection is the most common cause of early spontaneous preterm labour. Limitations of this study relate to the two time points (72 h and 96 h) selected for fetal delivery post-infection. This experimental design did not impact our ability to perform serial TDI assessment of fetal cardiac function; however it did limit our ability to determine time-dependent (i.e. 72 h vs. 96 h) changes in fetal infection and inflammation.

These novel findings are of interest as they provide evidence from a large animal model of pregnancy to demonstrate the ability of *C. albicans* to rapidly and comprehensively infect the fetus, causing significant pathological changes in the fetal lung. No significant changes in cytokine of chemokine transcript expression were detected in the chorioamnion, which is in keeping with our earlier work with *C. albicans* in mid-gestation sheep pregnancy[28].

Histological analysis of fetal skin revealed invasion of hyphal structures deep into the dermis. Surprisingly, the *C. albicans*-infected fetal skin exhibited comparatively little obvious changes in terms of immunocyte infiltration in H&E sections and only minor changes in cytokine and chemokine expression. These observations are in contrast to fetal ovine skin

responses to *C. albicans* in mid-gestation pregnancy[6], and also to stimulation with *E.coli* LPS in instrumented fetal sheep at a similar gestational age.[23] Although this difference may be due to differences in the length of agonist exposure (at least in regards to LPS-stimulation), it is also possible that the fetal skin is partly deficient in its ability to mount a pro-inflammatory response to some microorganisms, such as *C. albicans*, in early pregnancy. Such a conclusion is in keeping with the observation that fetal immunological capacity develops with increasing gestational age.[22]

Of particular interest, is the finding that it is feasible to perform real-time visualization of the effects of systemic infection and inflammation on the developing heart with non-invasive TDI ultrasound analysis. Intrauterine *C. albicans* infection in the fetus is rare in clinical practice, although there are data to suggest that its impact may be underreported.[6] Nevertheless, these experiments show that intrauterine *C. albicans* causes rapid and systemic fetal infection in association with fetal cardiac dysfunction that is detectable by TDI. It is possible that the cardiac findings were due to overwhelming fetal sepsis. With diastolic dysfunction identified at 24 h, future studies would need to include groups of fetuses delivered for necroscopy and infection analysis at a selection of earlier time-points in order to determine whether the pathological changes in cardiac function were in response to systemic fetal inflammation or sepsis.

Our results demonstrate that the study of the mechanisms leading to the persistent increased cardiovascular risk seen in children and adults born preterm is feasible. More importantly, development of non-invasive diagnostic tests for fetal infection and studies of therapies to improve cardiac function may be possible. Translating this work into clinical practice is a goal of future studies.

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TABLES

Table 1

	T0 (mean SD)	T24 (mean SD)	P	T72 (mean SD)	P
MV S’	4.74 (1.06)	4.99 (0.87)	0.112	6.27 (1.85)	0.022 *
MV E’	5.23 (1.42)	5.31 (1.88)	0.099	7.48 (2.45)	0.206
MV A’	7.75 (1.63)	10.6 (2.59)	0.053	10.37 (2.52)	0.093
MV EA’	0.67 (0.09)	0.5 (0.12)	0.084	0.73 (0.17)	0.561
IV S’	4.33 (0.86)	5.15 (0.30)	0.253	5.77 (1.41)	0.050*
IV E’	4.94 (1.41)	3.9 (1.28)	0.018*	6.49 (1.58)	0.236
IV A’	6.63 (1.17)	6.88 (2.17)	0.851	8.91(1.27)	0.023 *
IV EA’	0.75 (0.19)	0.57 (0.05)	0.016*	0.73 (0.15)	0.803
TV S’	6.04 (1.06)	6.71 (2.47)	0.982	6.12 (0.93)	0.888
TV E’	5.79 (0.95)	6.58 (1.33)	0.623	6.95 (1.02)	0.019 *
TV A’	8.87 (1.42)	9.75 (2.05)	0.909	9.36 (2.08)	0.379
TV EA’	0.65 (0.04)	0.68 (0.07)	0.169	0.76 (0.15)	0.166

Table 1: Mean (standard deviation) peak annular Systolic (S’), Early Diastolic (E’) and Late Diastolic (A’) velocities and E’/A’ Ratio at Left annulus (MV), Right annulus (TV) and Interventricular Septum (IV).

FIGURE LEGENDS

Figure 1: Representative images of histological analysis. Panel **i**, confocal images fetal skin from *C. albicans* infected fetus. Hyphae probed with *C. albicans*-specific primary antibody (green) with DAPI nuclear staining (blue). Panel **ii**, H&E images of fetal skin from control (A) and *C. albicans*-exposed (B) animals. Panel **iii**, H&E images of perfusion-fixed fetal lung from control (A) and *C. albicans*-exposed (B) animals.

Figure 2: Mean concentration of TNF-α in amniotic fluid (ng/mL). Error bars represent 1 SD. h, hours post-*C. albicans* exposure; Ctrl, control. *, $p<0.05$, vs. control.

Figure 3: Mean fold change in cytokine / chemokine transcript expression in *C. albicans*-exposed tissues (red bars), relative to control tissues (blue bars). A, fetal lung; B, fetal liver; and C, fetal myocardium. Error bars represent 1 SD. *, $p<0.05$, vs. control.

Figure 4: Diagram representing normal PW-TDI wave form representing mechanical events in cardiac cycle and summarizing changes seen in peak annular Systolic (S'), Early Diastolic (E') and Late Diastolic (E'/A' Ratio at Left annulus (MV), Right annulus (TV) and Interventricular Septum (IV) in fetal sheep at 24 hours and 72 hours after intra-amniotic injection of *C. albicans*.

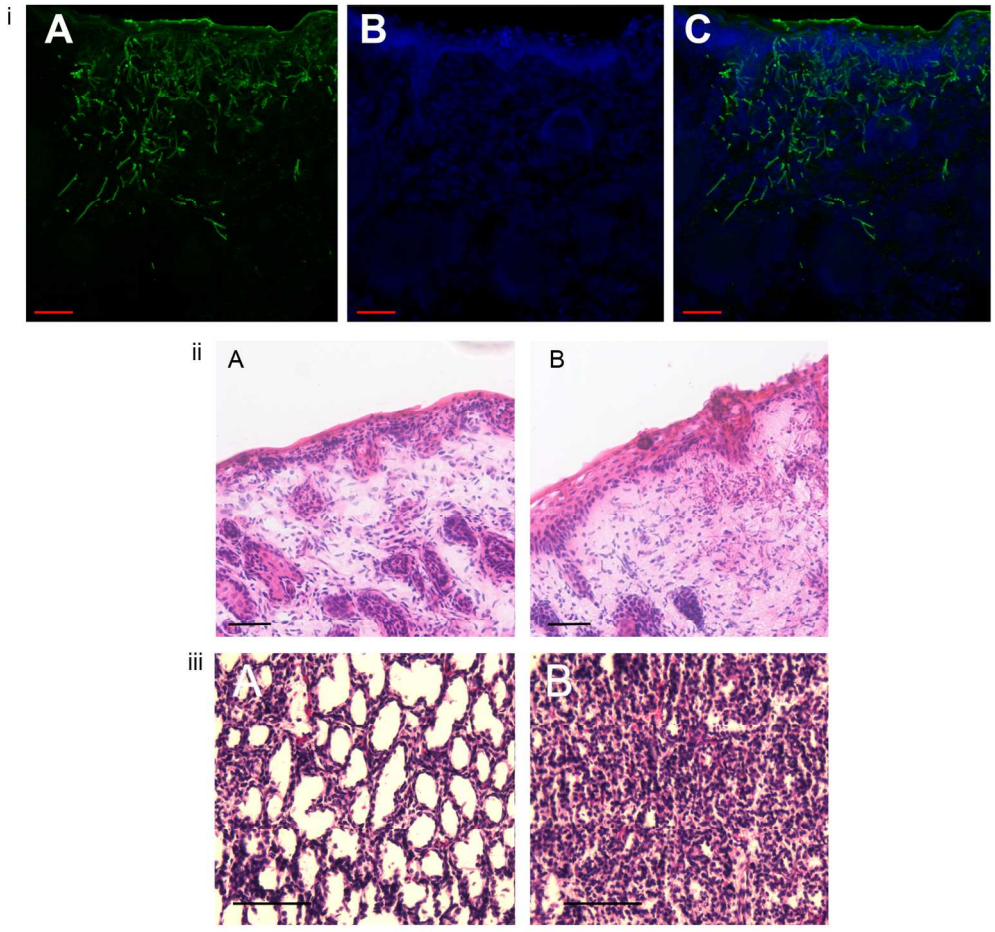


Figure 1: Representative images of histological analysis. Panel i, confocal images fetal skin from C. albicans infected fetus. Hyphae probed with C. albicans-specific primary antibody (green) with DAPI nuclear staining (blue). Panel ii, H&E images of fetal skin from control (A) and C. albicans-exposed (B) animals. Panel iii, H&E images of perfusion-fixed fetal lung from control (A) and C. albicans-exposed (B) animals.
147x138mm (300 x 300 DPI)

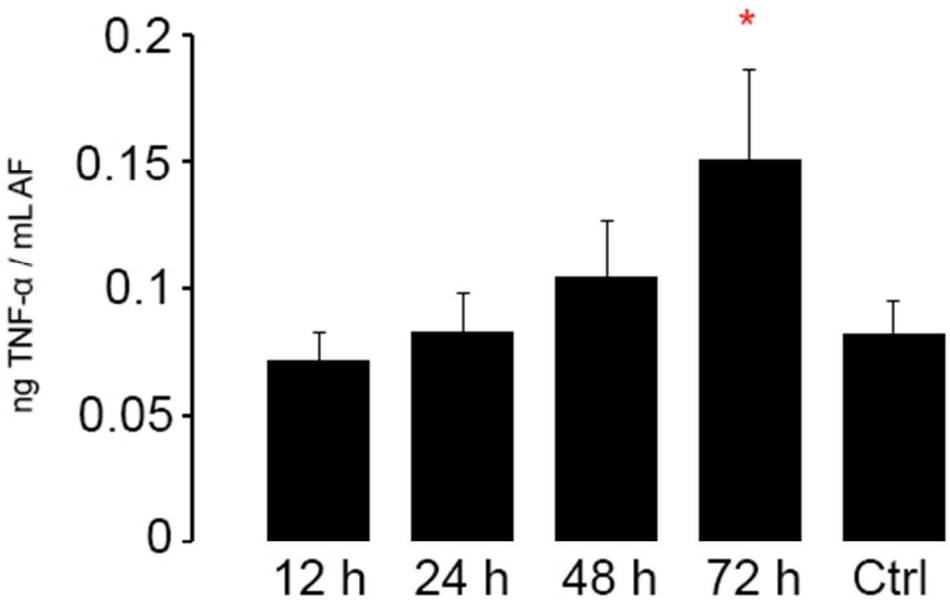
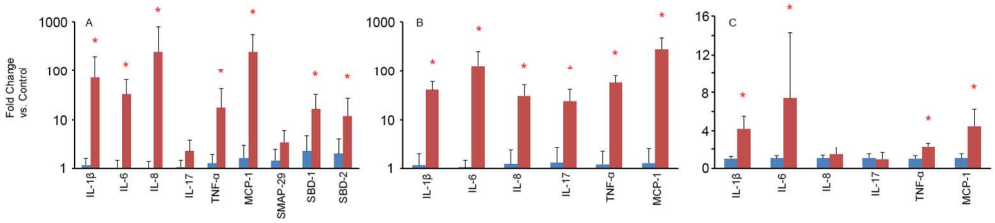


Figure 2: Mean concentration of TNF-α in amniotic fluid (ng/mL). Error bars represent 1 SD. h, hours post-C. albicans exposure; Ctrl, control. *, p<0.05, vs. control.
43x27mm (300 x 300 DPI)



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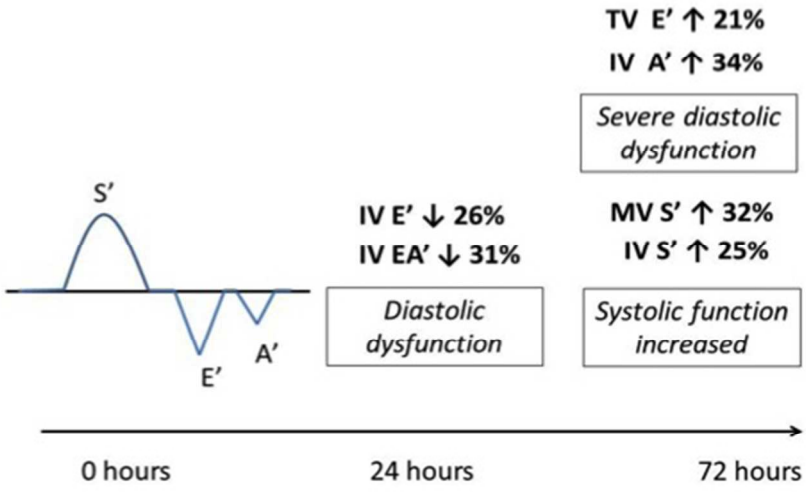
131x31mm (300 x 300 DPI)



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220x124mm (72 x 72 DPI)